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An Investigation of Epigenetic Contributions to Inter-animal and Age Dependent Variation in the Bovine Innate Immune Response.

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AN INVESTIGATION OF EPIGENETIC CONTRIBUTIONS TO INTER-ANIMAL
AND AGE DEPENDENT VARIATION IN THE BOVINE INNATE IMMUNE
RESPONSE

A Dissertation Presented

by

Benjamin Green

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of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Animal, Nutrition, and Food Sciences

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Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, specializing in Animal, Nutrition, and Food Science.

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ABSTRACT

Mastitis represents a major issue within the dairy industry responsible for economic loss via decreased animal productivity and associated veterinary costs. Currently, there is a push to identify a phenotypic innate immune response that will yield dairy cows with an enhanced resistance to mastitis. Bovine dermal fibroblasts were used as a cell model to measure the response of individuals to Gram-negative bacterial stimuli through the TLR4 signaling pathway. Fibroblast cultures were isolated from 15 dairy heifers at 5, 11, and 16 months of age in order to determine the variability in responsiveness to LPS as well as to monitor the development of the innate immune response in calves. These individuals displayed a large range in response to LPS as measured by IL-8 production. In addition, response within individuals increased dramatically with age.

To determine the cause behind this, DNA methylation was investigated as a potential player in the variation in response described both within an individual over time as well as across individuals. Fibroblast exposure to 5-aza-2'-deoxycytidine, a DNA demethylating agent, increased the cellular response to LPS, but more so in cultures that had previously displayed low responding phenotypes. This suggested that DNA methylation acted as an inhibitor of the innate immune response, and may be responsible for some degree of the variation seen in the LPS response. To determine the effect of epigenetic factors on this response, microarray analysis was conducted on RNA isolated from cells either having been epigenetically modified (DNA demethylation and histone hyperacetylation) or without undergoing any epigenetic treatment. This analysis identified 1,758 genes with altered expression due to epigenetic modification.

To focus on DNA methylation's role, methylated CpG island recovery assay (MIRA-Seq) libraries were created from fibroblasts to investigate differential methylation from a group of the same individuals sampled at 5 and 16 months of age. In addition, transcriptomic data were generated by RNA-Seq from fibroblasts collected from the young and older samples and exposed to LPS for 0, 2, and 8 hours to characterize age-associated changes in the innate immune response. Cultures from older animals were much more responsive to LPS as indicated by greater expression of IL-8, IL-6, TNF- α , and CCL20 at various times in response to LPS. TLR4 and CD14 were more highly expressed in older cultures, suggesting these fibroblasts are more able to detect the presence of LPS. Analysis of the bovine fibroblast methylome revealed methylation with remarkable stability except for 20 regions along the genome undergoing major shifts due to age. Similar data were collected from fibroblasts isolated from different individuals displaying either a low or high responding phenotype resulting in 843 regions with differential methylation between groups. This suggests that DNA methylation may be playing a role in both the age-dependent and between animal differential responses to LPS, and also gives the first in depth look at the bovine fibroblast methylome and its stability over time.

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CHAPTER 1:
Literature Review

1.1 Mastitis and its Economic Impact

Inflammation of the mammary gland, or mastitis, is a disease of great overall concern within the dairy industry. A 2007 survey conducted by the United States Department of Agriculture (USDA) found that a measurement of farmer reported clinical cases yielded an average of 18.2% of cows per farm contracting mastitis, with 89.9% of these infected cows receiving antibiotic treatment (USDA 2007). A case of clinical mastitis caused by a Gram-positive pathogen has an average cost of \$133.73 USD to the producer while a case caused by a Gram-negative pathogen incurs a cost of \$211.03 USD (Cha, Bar et al. 2011). These costs are most frequently attributed to lower milk production from infected animals, veterinary costs, and premature culling of animals from the herd (Schukken, Hertl et al. 2009, Cha, Hertl et al. 2013).

In addition to animal health and welfare concerns, mastitis often leads to a lower quality milk product. A cow with an infected mammary gland will produce milk with an increased somatic cell count (SCC), which in turn increases the proteolytic activity within the gland (Hinz, Larsen et al. 2012) through activation of enzymes such as collagenase, cathepsins and elastase (Moussaoui, Michelutti et al. 2002). High SCC milk generally has a shorter shelf life before scoring poorly for qualities such as rancidity and off-flavor (Ma, Ryan et al. 2000). In this, mastitis has a negative impact on all involved, from the infected animal, to the producer, and eventually the consumer. Keeping mastitis incidence low through a better understanding of factors leading to an animal's increased susceptibility to disease would greatly benefit the US dairy industry both at the production and consumer levels.

1.2 Causative Agents of Bovine Mastitis

Mastitis is caused by a wide array of pathogens. The most commonly diagnosed etiological agents of mastitis in the United States are the bacteria *Staphylococcus aureus*, other coagulase-negative *Staphylococcus* species (CNS), *Streptococcus uberis*, and *Escherichia coli* (Contreras and Rodríguez 2011). Bacteria causing mastitis are usually classified as either environmental or contagious in nature. However unique classification for an individual pathogen has proven difficult and there is often disagreement in cataloging (Fernández, Barreal et al. 2013).

Staphylococcus aureus is a Gram-positive cocci that often elicits a comparably muted immune response in the mammary gland when compared to other pathogens (Lee, Bannerman et al. 2006). *Staphylococcus aureus* often manages to cause a subclinical, chronic infection that may become a lifelong issue for an infected animal (Sutra and Poutrel 1994). The longer an infection is allowed to persist prior to first treatment, the more difficult it becomes to clear, prioritizing prevention before treatment of mastitis (Owens, Ray et al. 1997, Barkema, Schukken et al. 2006). Mastitis associated with *S. aureus* generally leads to an increased milk SCC, as well as production of pro-inflammatory cytokines, though at low levels when compared to acute infections (Bannerman, Paape et al. 2004).

Escherichia coli, in contrast to *S. aureus*, is a Gram-negative rod that elicits a rapid, acute immune response within 12-24 hours of entry into the mammary gland. *Escherichia coli* infections lead to a drastic increase in pro-inflammatory cytokines, milk SCC, and tissue damage within the gland as measured by milk blood serum

albumin (BSA) levels (Bannerman, Paape et al. 2004). While antimicrobial treatment of coliform mastitis has shown in some instances to be effective (Bengtsson, Unnerstad et al. 2009), symptoms of infection are often self-resolving and many producers choose to avoid treatment and allow for natural clearance except in severe cases.

Comparing the two pathogens, *E. coli* leads to the production of large quantities of tumor necrosis factor- α (TNF- α) within the gland while *S. aureus* causes only minor increases in its production. This likely plays a major role in the clinical differences seen in the pathogenicity of the two microbes as TNF- α is considered an important pyrogen and response initiator (Wellnitz and Bruckmaier 2012). In addition to TNF- α production, *E. coli* mastitis leads to higher levels of interleukin-6 (IL-6), and IL-8 in the gland than *S. aureus* (Griesbeck-Zilch, Meyer et al. 2008). IL-6 is important in the progression of inflammation while IL-8 acts as a chemotactic agent for neutrophils, key players in the resolution of infections. The variability in response to *E. coli* and *S. aureus* can be linked to how each microbe is recognized by the innate immune response system and its subsequent activation.

1.3 Innate Immunity and Toll-like Receptors

The innate immune response acts as the first line of defense against an invading pathogen. Activation of this system results in a rapid, generalized response against infection. Immunostimulation occurs following recognition of a pathogen associated molecular pattern (PAMP), a repeating motif common across many microbes. The PAMP's are recognized by pattern recognition receptors (PRR)

including the membrane bound family of proteins, Toll-like receptors (TLR) (Beutler, Hoebe et al. 2003). Multiple TLRs exist on a cell membrane (or membrane bound within the endosome) with each responding to a different PAMP. For example, TLR4 recognizes lipopolysaccharide (LPS, also known as endotoxin) of Gram-negative microbes while TLR2 (which forms a heterodimer with TLR1 or 6) responds to multiple Gram-positive PAMPs including peptidoglycan (PGN) and lipoteichoic acid (LTA) (Ozinsky, Underhill et al. 2000). These were recently confirmed as TLR2 PAMPs by neutralization of the TLR2 transmembrane domain, which inhibited response to these molecules, but not to LPS, the TLR4 agonist (Fink, Reuven et al. 2013).

Of the host proteins involved in TLR activation and subsequent signal transduction, several play important roles in modulating the ability of the receptors to bind to their associated PAMP. Focusing on the response to LPS, a pro-inflammatory response is induced by the coordinated binding of endotoxin to four principle proteins: LPS binding protein (LBP), cluster of differentiation (CD)14, lymphocyte antigen 96 (MD2), and TLR4 for which there is a low threshold of activation before signaling occurs (Jerala 2007, Panter and Jerala 2011). Following stimulation of a TLR, the response may follow one of two paths, either involving myeloid differentiation factor 88 (MyD88) or a MyD88-independent response. Whether MyD88 is involved in the TLR4 response appears to depend on which of the binding proteins are involved in the reception of LPS (Zughaier, Zimmer et al. 2005). The MyD88-dependent response leads to a rapid activation of nuclear factor kappa light chain enhancer of activated B

cells (NF- κ B) an important transcription factor that allows for immune related gene transcription. In contrast, the MyD88-independent pathway leads to production of interferon (IFN)- β and a more moderate, delayed NF- κ B activation (Kawai, Takeuchi et al. 2001, Günel 2012).

Focusing on the MyD88-dependent response pathway, following association of MyD88 with the Toll IL-1 Region (TIR) domain of the TLR receptor, a downstream cascade of proteins are involved in NF- κ B activation. Briefly, isomers of the interleukin receptor-associated kinase (IRAK) family of proteins are recruited, specifically IRAK1, which is in turn phosphorylated by IRAK4 (Li, Strelow et al. 2002, Napetschnig and Wu 2013). IRAK1 phosphorylation leads to the recruitment of TNF receptor associated factor (TRAF)6 and the activation of Mitogen-activated protein kinase kinase kinase (MAP3K)7 (Beutler 2009). MAP3K7 then marks I κ B, which acts as an NF- κ B inhibitor, for ubiquitination and subsequent destruction in the proteasome. This final step allows for the translocation of NF- κ B to the nucleus beginning transcriptional activation of cytokines (Hayden and Ghosh 2008).

The milieu of pro-inflammatory cytokines up-regulated following NF- κ B translocation prime the immune system for a rapid, though non-specific response to invading pathogens. Under normal conditions within the mammary gland, macrophages are the predominant leukocyte present (Koess and Hamann 2008). Following infection however, an influx of neutrophils dramatically increases the number of somatic cells present in addition to altering cell proportions (95% neutrophil) playing an important role in pathogen clearance (Burvenich, Paape et al. 1994, Paape, Shafer-Weaver et al.

2002). The key cell type implicated in the up-regulation of pro-inflammatory cytokines and this subsequent influx of neutrophils are mammary epithelial cells as demonstrated in goats (Brenaut, Lefevre et al. 2014). The cytokines involved in this process include IL-8, IL-6, IL-1 β , and TNF- α , which are all products of NF- κ B activation (Persson Waller, Colditz et al. 2003). Upon infection, cells throughout the gland upregulate expression of important factors including IL-8 and TNF- α within 12 hours of exposure, highlighting the rapid response (Rinaldi, Li et al. 2010). Following migration of neutrophils into the mammary gland, phagocytic bacterial clearance occurs. Functional deficiencies in neutrophil activity lead to immunodeficiency and critical gaps in the processing of bacteria (Rinaldi, Moroni et al. 2008). Clearly, the innate immune response plays a major role in the resolution of mastitis within the bovine mammary gland. Understanding the process of pathogen recognition within the gland by TLR4 up to the influx of critical leukocytes and bacterial clearance is paramount to lowering the rate of mastitis in the United States and increasing animal welfare and livestock productivity.

1.4 What is the optimal phenotypic response to bovine mastitis?

A better understanding of what the ideal innate immune phenotype may be for a dairy cow along with the ability to manipulate the population towards this ideal both effectively and naturally is paramount to the dairy industry. Effective pathogen clearance on a consistent basis is desirable; however, this must be accomplished with minimal collateral damage to the host. There are currently two differing opinions within

the field of mastitis research as to whether a rapid, strong immune response is best or if a more mild reaction is sufficient in pathogen clearance.

Those favoring a strong, rapid response often cite studies that illustrate the importance of robust cytokine production, in which failure to produce sufficient levels of IL-8, IL-1 β , and TNF- α led to an ineffective clearance of the pathogen (Bannerman 2009). With this in mind, several studies have explored the use of recombinant bovine cytokines as a treatment for mastitis, with the idea that a higher concentration of these pro-inflammatory proteins is desirable. Among those investigated were IFN- γ (Sordillo and Babiuk 1991), IL-1 and IL-2 (Daley, Williams et al. 1993), as well as IL-8 (Takahashi, Komatsu et al. 2005), which were all reported to either improve clearance of or limit microbial infection. However, these do not act as prophylactics and in working as a treatment rather than preventing mastitis cases, have not made a market impact. Cows have also been reported to be most susceptible to *E. coli* infections during periods of lactation associated with immunosuppression (De Schepper, De Ketelaere et al. 2008), suggesting a limited response would in fact elevate mastitis rates.

In contrast, others have presented data suggesting that a rapid, intense immune response unnecessarily leads to host tissue damage while providing no advantage in pathogen clearance. Cows selected as displaying either a low or high phenotypic innate immune response based on *in vitro* data were able to clear an intramammary *E. coli* infection similarly. Importantly, the low responding cows returned to baseline milk production levels sooner, and had higher quality milk throughout the trial as measured

by SCC and blood serum albumin (BSA) levels (Kandasamy, Green et al. 2011).

Artificial reduction of the innate immune response also has proven to demonstrate that a milder immune response may be beneficial (Sipka, Gurjar et al. 2013). In that study, cows were experimentally exposed to *E. coli* in three separate quarters. One quarter remained untreated, one received antibiotics, while the third received antibiotics along with a steroid. Mammary gland histology 24 hours post challenge showed that the quarter receiving the steroid treatment had a lower neutrophil influx than other challenged glands, however this gland also displayed the highest rate of bacterial clearance (Sipka, Gurjar et al. 2013). Though more thorough research on the use of steroids as a mastitis therapeutic is required, the data suggest that a limited immune response is sufficient in pathogen clearance. A similar conclusion may be drawn from work focusing on differing strains of *S. uberis*, of which one was a putative host-adapted pathogen causing chronic, persistent infections in cows in contrast to a second strain causing only transient infections. While the transient, non-adapted strain elicited only a mild response from cows experimentally exposed, the microbe was eliminated from the gland rapidly. The host-adapted strain however caused a large increase in cytokine levels within the gland, but was not cleared within the 13 days of monitoring (Tassi, McNeilly et al. 2013). Though these data focus on strain differences rather than variable responses from individuals, it is compelling to note that even though both pathogens grew similarly in milk *in vitro*, the pathogen eliciting a weak response was cleared rapidly, caused lower clinical scores in animals, and also reduced milk yields from animals at a much lower level.

The differing opinions on the optimal phenotypic response in dairy cattle grows even more complicated when one takes into account that these studies focused on the clearance of a single pathogen. Mastitis within the dairy industry is caused by a wide array of microbes that may each be optimally cleared by a response of varying magnitude. However, in the opinion of the author, the dairy industry should pursue a strategy that moves cows towards a more mild response, in which recognition of the microbe and subsequent activation of the innate immune response is sufficient for clearance while minimizing clinical signs. This strategy should allow for lowered mastitis rates, while also maximizing profits during an infection through increased milk quality and higher milk production.

1.5 Mastitis and Genetic Selection

Manipulation of population genetics through artificial selection has been employed for resistance to mastitis for many generations either directly or indirectly (cows with an increased frequency of infection and lower productivity are more likely to be culled). Using index measurements directly cataloging and recording mastitis events has in question has reduced clinical mastitis cases within three generations of implementing direct selection (Heringstad, Klemetsdal et al. 2007). This study also noted however, that heritability of mastitis resistance was still extremely low ($h^2=0.03$) and this strategy is labor intensive and likely unrealistic for large populations outside of a research setting. Estimates blocking for period of lactation surveyed were only able to increase heritability values to between 0.05 and 0.07 (Hinrichs, Stamer et al. 2005)

suggesting that genetic components may be playing a smaller role in mastitis susceptibility than previously thought.

Indirect selection against susceptibility to clinical mastitis utilizes the measurement of traits that may be correlated to disease. To indirectly select for improved herd health, researchers have most frequently focused on using either milk SCC or milk somatic cell score (SCS) as a selection parameter within studies. These traits offer several advantages including a moderate to strong correlation to mastitis, and better heritability values than direct selection using mastitis incidence. Studies using SCC and SCS found correlations between these values and clinical mastitis ranging from 0.6 to 0.88 and with heritability values from 0.04 to 0.11 (Vallimont, Dechow et al. 2009, Pritchard, Coffey et al. 2013). While these results appear promising at first glance, there are several issues with indirect selection using SCC and SCS that are limiting factors. On most working farms an SCC measurement is only taken monthly which would likely miss detecting any increased values from a brief, acute, mastitis event (Detilleux 2009). In addition, while a low SCC value may indicate a slight resistance to mastitis and would increase milk quality for the producer, selection for extremely low SCCs within a herd may have a deleterious effect as moderate levels appear to be necessary for disease resistance (Detilleux 2004).

A separate approach utilizes specific genetic markers to aid in the selection of animals with a superior phenotype. This technique runs into several similar issues as trait-associated selection, namely, difficulty in determining a phenotype to be used for genetic marker studies that is suitably linked to clinical mastitis susceptibility. Many

genome wide association studies (GWAS) have been performed using SCC and SCS as their phenotype, identifying multiple genomic regions associated with these traits (for a comprehensive list: (Pighetti and Elliott 2011)). A meta-analysis examining QTLs relating to SCC or clinical mastitis susceptibility found that only several of the regions identified were common across multiple studies (Khatkar, Thomson et al. 2004) and these were often near genomic coordinates previously linked to milk production which has been shown to have a negative correlation to mastitis incidence. This data seems to suggest there is no clear correspondence between QTL locations and mastitis susceptibility. While genetic variation likely plays a role in differential immune responses between individual cows and their subsequent susceptibility to mastitis, the relative inability of genetic testing to account for these differences suggests another layer of gene regulation may be involved.

1.6 Epigenetics

Gene regulation is frequently referenced solely in terms of gene transcription controlled by proteins that interact directly with DNA. However, DNA itself may be covalently modified in ways that affect gene transcription and therefore play a role in the biological processes of the cell on an important level (Verdone, Caserta et al. 2005, Ehrlich and Lacey 2013). These changes in DNA are collectively referred to as epigenetic modifications. The study of epigenetics focuses on changes occurring in DNA that does not involve variation in the primary sequence of genes, therefore does not involve changes in A, G, T, C base pairing. While maintaining the primary

sequence, there are several other mechanisms that play an important role in gene transcription and cell regulation.

1.7 Histone Modifications

Packaging of DNA within the nucleus plays several important roles in cellular function. First is the interplay between the size of the genetic material within a cell and the limited space allowed within the nucleus. In addition, DNA packaging also acts as a key regulatory step in gene transcription as mediator of access. The nucleosome is comprised of several core proteins known as histones. These histones, H2A, H2B, H3, and H4 form an octamer comprised of two H2A-H2B dimers and an H3-H4 tetramer. A single octamer may interact with a 147bp segment of DNA, with a string of these interactions occurring along a strand of DNA, with each nucleosome structure separated by approximately 50bp of DNA (Kouzarides 2007).

The genome may variably be found in two distinct states at any given time in terms of its packaging status. Transcriptionally active euchromatin is open and loosely packaged while silent, inactive heterochromatin is tightly wound around histone proteins. This mechanistically occurs as a simple physical barrier. Initiation of transcription may be blocked as transcription factors will not have access to binding sites bound to histones, and even after transcription has begun histones may block elongation of engaged polymerases (Portela and Esteller 2010).

While histone modifications may include methylation, phosphorylation, and ubiquitination, this review will focus on histone acetylation. Gene expression has been

correlated with histone acetylation levels as high levels of acetylation often characterizes euchromatin, while conversely, heterochromatin is generally linked to low histone acetylation levels (Li, Carey et al. 2007). This association however is an oversimplified view of the complexity of histone regulation as different histones within the same nucleosome, or histones of nearby nucleosomes may be undergoing modifications antagonistic to one another. Research has shown that histone modifications appear to occur as a complex “cross-talk” within and across these octamers to allow for fine tuned control of gene transcription (Wang, Zang et al. 2008).

Histone acetylation is controlled by the interplay between histone acetyltransferase (HAT) enzymes and histone deacetylases (HDAC) enzymes that link or remove an acetyl group from the terminal lysine residues of histone tails, respectively (Allfrey, Faulkner et al. 1964). Multiple HAT enzymes have been identified and classified into groups, predominantly the Gcn5 and MYST families, based upon their catalytic domain (Kimura, Matsubara et al. 2005). HDAC enzymes form similar subsets of classes, each recognizing and reacting to different acetyl group markers (Ekwall 2005).

1.8 DNA Methylation

DNA methylation is the stable conversion of cytosine residues into 5-methylcytosine, in which a single methyl group is covalently bonded to the five-position of the six-carbon ring. This phenomenon in vertebrates occurs only in cytosine residues that are immediately followed by a guanine, resulting in a CG dinucleotide,

often referenced as a CpG. DNA methylation plays a major role in mammalian development through stable regulation of important genes involved in embryogenesis. This regulation manifests itself frequently as genomic imprinting, in which a subset of genes display monoallelic expression dependent on parent-of-origin (Barlow 2011). DNA methylation also plays a role in cell differentiation and tissue specificity, much of which is becoming more clearly understood as methylation profiles are being created from different tissue types (Gordon, Joo et al. 2012, Herzog, Galvez et al. 2013). In addition, methylation acts as an important regulatory element in genome-wide control of gene transcription and *de novo* addition of methyl marks early in embryogenesis has major implications on normal development. Cytosine residues are converted to 5-methylcytosine by DNA methyltransferase 3A (DNMT3A) and 3B, while methylation patterns are maintained by DNMT1 in cooperation with Ubiquitin-like, containing PHD and RING finger domains (UHRF)-1 (Okano, Bell et al. 1999, Chen and Li 2006). In mammals, a large proportion (ranging from 60-80% depending upon tissue type) of CpG dinucleotides are methylated, however, regions known as CpG islands which are stretches of DNA with a high density of CpGs (~55% or higher) are generally hypomethylated (Takai and Jones 2002, Lister, Pelizzola et al. 2009). These CpG islands are often associated with promoter regions of genes, though ‘orphan’ islands may be found in intergenic regions and are often more heavily methylated than their promoter related counterparts (Illingworth, Gruenewald-Schneider et al. 2010).

Maintenance of hypomethylation at these promoter CpG islands is greatly influenced by the continued presence of bound transcription factors blocking access of

DNMT proteins to the DNA. This principle was demonstrated through the accrument of methylation at a previously hypomethylated promoter region following the truncation of the transcription factor binding sequence (Macleod, Charlton et al. 1994). While hypomethylation is the dominant status of CpG rich promoter regions, it is not infrequent that promoters, especially those containing lower CpG density find themselves methylated to induce gene silencing. In these cases, it appears as though DNMT3A and 3B work in partnership with other epigenetic mediators such as histone deacetylase (HDAC) proteins and the nucleosome organizer lymphoid-specific helicase (LSH) to ensure methylation of appropriate regions (Myant and Stancheva 2008).

Following *de novo* methylation, proper maintenance of these methyl marks becomes paramount. To accomplish this, UHRF1 identifies hemi-methylated cytosine residues following mitosis, recruits DNMT1, acting as the catalytic methyltransferase enzyme, and also flips the methylcytosine from the parent strand out of the double helix to allow access to the unmethylated residue on the daughter strand (Arita, Ariyoshi et al. 2008, Hashimoto, Horton et al. 2008). DNMT1 and UHRF1 are most abundant during the S phase of mitosis and associate with the protein proliferating cell nuclear antigen (PCNA) that localizes along the replication fork (Chuang, Ian et al. 1997). These proteins, acting in cooperation, work to ensure that methylation marks originally laid down during development are faithfully maintained.

1.9 Epigenomic Sequencing Techniques

There are several popular methods currently being employed for high throughput sequencing to map DNA methylation on a genome wide scale. Each offers distinct advantages and disadvantages, with whole genome bisulfite sequencing, reduced representation bisulfite sequencing (RRBS), and methylated DNA immunoprecipitation (MeDIP, among other similar techniques) being available to bovine researchers, as well as the microarray based Infinium chip series, which in contrast is specifically designed for human analysis.

Whole genome bisulfite sequencing utilizes the chemically induced creation of single nucleotide polymorphisms through exposure of genomic DNA to sodium bisulfite, which selectively converts unmethylated cytosines into uracil while methylated CpGs are protected (Clark, Statham et al. 2006). Following selective conversion of the genome, sequencing allows for the comparison of converted to unconverted DNA, giving methylation status of a sample at a single base pair resolution. This gives whole genome bisulfite sequencing unquestionable power in discerning small differences in methylation. However, while this technique provides the most information of any sequencing method described, it also requires extremely deep sequencing with over 1 billion reads per sample required (Lister, Pelizzola et al. 2009). This can often exclude whole genome bisulfite sequencing as an option due to the prohibitive cost and logistical difficulty associated with the production of such large data sets.

In a similar manner to whole genome bisulfite sequencing, RRBS offers mapping of DNA methylation at the resolution of a single base pair. In contrast to whole genome sequencing however, RRBS offers investigation of a more limited portion of the genome, frequently focusing only on promoter regions, or genes with functional interest. This is accomplished by selectively enriching for genomic regions with moderate to high densities of CpG residues by using the restriction enzyme MspI that cleaves at 5'-CCGG-3' sites. Following cleavage, size selection of fragments allows the investigator to selectively isolate small stretches of DNA with CpG sites at the terminal ends. These fragments are then bisulfite converted, allowing for similar analysis to whole genome bisulfite sequencing, however by focusing on less of the genome, costs are much more manageable (Meissner, Gnirke et al. 2005). Recent publications have made advances in the amount of the genome that may effectively be analyzed and researchers have reported obtaining 76.7% of CpG islands, 54.9% of CpG island shores and 52.2% of core promoters in the human genome covered with at least 3 CpG sites per region (Lee, Jin et al. 2014).

DNA methylation may also be analyzed by the creation of a DNA library that has been enriched for methylated regions via the use of antibodies raised against 5-methylcytosine. Following enrichment for methylation, high throughput sequencing allows for the sequenced regions to be mapped back to the genome of interest to visualize the methylome (Weber, Davies et al. 2005). These mapped reads will vary in length depending upon the type of sequencing chemistry used, but frequently will average at approximately 100 bp giving the investigator a lower resolution to discern

differences in methylation status. While unable to identify methylation status of a single CpG, the antibody based method does allow for quantification of methylation levels within a region as a segment of DNA with higher methylation levels will be isolated at a higher rate. In this, read count now may be used to compare samples for differentially methylated regions (DMRs). Other similar techniques have been described, each focusing on the use of positive selection to enrich a library for methylated DNA. For example, rather than using antibodies against 5-methylcytosine, methylated CpG island recovery assay sequencing (MIRA-Seq) uses a dual methyl binding domain protein construct (MBD2b/MBD3L1) to isolate methylated DNA with a higher affinity than MeDIP (Mitchell, DeAngelis et al. 2011).

Finally, the Illumina Infinium microarray series utilizes DNA chip technology to identify methylated CpGs at a single base pair resolution with coverage of 99% of annotated genes and 96% of identified CpG islands within the human genome. The Infinium chip is able to produce data for 485,000 CpG dinucleotides, comparable in output with whole genome bisulfite sequencing. A direct comparison of the two techniques produced results with a strong correlation of $r^2=0.95$ (Bibikova, Barnes et al. 2011). The array offers multiple probes per gene and CpG island of interest, which when averaged offers reliable values. However, as with many microarray-based assays, annotation of genomic region and coordinates is frequently incorrect, placing a certain level of difficulty on the investigator in discerning correct values from errors (Wilhelm-Benartzi, Koestler et al. 2013).

Each of the sequencing techniques described here offers distinct advantages along with several caveats. Understanding the interplay between these is an important factor when designing studies of the methylome. The resolution of the data, the amount of the genome investigated, and ultimately the cost of sequencing must all be taken into account for any given experiment. In addition, shortcomings for the technology employed may at times be overcome by advanced planning in design. For example, while MeDIP and MIRA-Seq technologies offer a low resolution that would appear to exclude any conclusions of minor differences in methylation from being inferred, using a robust sample size may overcome this issue (Bock, Tomazou et al. 2010). Overall, the recent advancement in sequencing technologies as they specifically pertain to genome-wide methylation studies, has greatly aided in the further understanding of DNA methylation and its role in disease.

1.10 Epigenetics and Disease

Within the past ten years, a growing body of evidence has linked epigenetic alterations and variation within a population to different disease states. This idea culminated in the belief that environmental stimuli present between conception and birth are playing a role in altering epigenetic marks that may lead to differential disease susceptibility (Gluckman, Hanson et al. 2005). The prenatal developmental period may be a time of phenotypic plasticity in response to fetal and maternal communication on environmental stresses that will shape the overall fitness of the offspring (Gluckman, Hanson et al. 2007).

Environmental assaults during pregnancy have been directly linked to alterations in the innate immune response following an LPS challenge. Exposure of pregnant rats to endotoxin severely limited the ability of the offspring to respond to LPS as a neonate (Hodyl, Krivanek et al. 2008). This phenomenon has also been demonstrated in mice, with the offspring exposed to endotoxin *in utero* displaying a stunted immune response 40 weeks after birth (Williams, Teeling et al. 2011). Similar research also demonstrated that rat pups exposed to LPS *in utero* had delays in the development of their innate immune response (Hodyl, Krivanek et al. 2007). While these environmental cues were directly linked to alterations in the immune response, a direct epigenetic change was not investigated in relation to the studies.

Studies working to directly link changes in epigenetic status to alterations in the immune response would appear to bridge the gap between environmental cues and differential innate immune response. Analysis of the methylation status of genes related to the innate immune response has shown that variability in the methylome may have a direct link to the production of certain cytokines. Hypomethylation of the IL-6 and IL-8 gene promoters has been linked to higher production of the cytokines and to chronic inflammation causing rheumatoid arthritis and periodontitis (Andia, de Oliveira et al. 2010, Ishida, Kobayashi et al. 2011). In addition to this, increasing histone acetylation in human intestinal epithelial cells was able to potentiate the cellular response to LPS as measured by IL-8 protein production (Angrisano, Pero et al. 2010). Components of the innate immune signaling pathway have also been shown to display differential expression due to variation in epigenetic status. DNA methylation has been linked to

alteration of TLR4, MD-2, and I κ B α expression in intestinal epithelial cells leading to differential response to LPS stimulation (Takahashi, Sugi et al. 2009, O'Gorman, Colleran et al. 2010, Vamadevan, Fukata et al. 2010).

The environmental cues investigated, differential epigenetic status discovered, and variable immune responses described do not carry much practical merit without links to disease susceptibility. In humans, maternal environment has been linked to the development of diabetes, hypertension, and asthma (Bousquet, Jacot et al. 2004, Gluckman, Hanson et al. 2005). Epigenetic factors have also increasingly been linked to bacterial infections and inflammation. Infections involving *Helicobacter pylori* have been linked to aberrant DNA methylation in gut mucosa due to the presence of the bacterium (Maekita, Nakazawa et al. 2006). In addition to *H. pylori*, bacterial induced alterations in DNA methylation levels have been seen following infections with *Escherichia coli* in uroepithelial cells (Tolg, Sabha et al. 2011), *Porphyromonas gingivalis* in gingival epithelial cells (Yin and Chung 2011), and *Campylobacter rectus* in the placental tissue of pregnant women (Bobetsis, Barros et al. 2007). The targeted alteration of DNA methylation following bacterial infection would suggest that this epigenetic modification is playing an important role in the susceptibility and outcome of infections within an individual.

1.11 Methylation and Age

Alterations in both global and site-specific DNA methylation in normal, healthy cells, as a function of age, have been reported in multiple studies (Bjornsson,

Sigurdsson et al. 2008, Christensen, Houseman et al. 2009). While developmental cues have been shown to play a major role in DNA methylation patterns, environmental stimuli early in life have also been linked to alterations in methylation levels. This is best exhibited in studies focusing on methylation status of monozygotic twins whom would have experienced identical intrauterine environments, but exhibit epigenetic divergence with age (Fraga, Ballestar et al. 2005). Comprehensive analysis of DNA methylation in tissues over time has been made possible by the Illumina Infinium 27K and 450K arrays (Sandoval, Heyn et al. 2011) that report methylation status down to a single CpG resolution at either all promoter sites or genome wide, respectively. Multiple studies conducted using this technology have provided a better understanding of DNA methylation changes over time including an age-associated increase in methylation at the promoters of genes associated with development that frequently have a high CpG density (Maegawa, Hinkal et al. 2010, Rakyan, Down et al. 2010), while regions of low CpG density undergo a loss of methylation (Heyn, Li et al. 2012, Hannum, Guinney et al. 2013).

An initial question posed following the discovery of age-associated changes in methylation was whether the changes seen were tissue specific in nature, as research suggested that changes occurring in one tissue subset were not seen in others. Specifically, data have been presented indicating that changes seen in blood samples were due more to a shift in proportions of cell type in whole blood rather than methodical alterations in DNA methylation levels (Langevin, Houseman et al. 2011, Houseman, Accomando et al. 2012). While this does not discredit the changes seen in a

pure cell population or a tissue sample made up of stable proportions, it does suggest that changes seen in one population do not necessarily help in drawing conclusions or validation of a different population.

Determining whether the population of cells in question is mixed and in what proportion different cells types are found is important and when done appropriately, DNA methylation status has been shown to be an effective predictor of age. Specifically, analysis of buccal sample DNA methylation status at three loci was able to accurately predict age (Bocklandt, Lin et al. 2011), while whole blood analysis using the 450K Infinium methylation chip has also showed promising results with age prediction (Hannum, Guinney et al. 2013). Importantly, recent data using peripheral blood DNA have suggested that alterations to the bulk of DNA methylation occurred in a pediatric population between the ages of 3 and 17 and these changes accounted for much of the variability seen in a separate adult population (Alisch, Barwick et al. 2012). In addition, a longitudinal study focusing on alterations of DNA methylation in monozygotic twins between birth and 18 months of age found major changes in the levels of methylation by 1.5 years of age (Martino, Loke et al. 2013). Taken together, these data suggest that age-associated changes in DNA methylation occur very early in life and could be playing a role in an individual's sensitivity to environmental stimuli.

As methylation has been shown to change with age, and age frequently has a strong correlation to disease risk, the obvious next question is whether altering methylation over time may be linked to disease incidence. While promoter regions in general have been reported to show relative stability in their methylation levels, a

recent study conducting epigenome wide analysis of monozygotic twins suggested that slight alterations in these regions might be linked to age-related phenotypic traits (Bell, Tsai et al. 2012). Genes that had previously been shown to undergo age-associated changes in methylation have been linked to the incidence of certain cancers, thus linking their methylation status with disease and indirectly implicating age in this process (Teschendorff, Menon et al. 2010). Similar results have been seen in other studies focusing on age-associated frailty as well as acute myeloid leukemia (Rakyan, Down et al. 2010, Bellizzi, D'Aquila et al. 2012).

What has been difficult to determine for those concerned with the alterations in DNA methylation levels with age and its association with varying disease states is whether aberrant methylation is the driving force behind disease or is a bystander in this process and is simply a result of the disease being present. What may make this question even more difficult to address is that for a given disease, alterations in methyl status may be present as both a passenger and a driver of clinical symptoms. This topic has been extensively reviewed in relation to cancer epigenomics (Kalari and Pfeifer 2010).

1.12 Unanswered Questions

Very little is currently known about the role of methylation within the bovine system. Only recently was the first complete map of the bovine methylome published for any cell or tissue type, with placental tissue as the main focus of that study (Su, Wang et al. 2014). In addition to our lack of knowledge about DNA methylation levels

across varying cell types, there are no studies currently available that investigate the role of methylation on cell physiology.

A high degree of variation exists within the Holstein population in the ability to recognize, respond to, and clear a pathogen following experimental *E. coli* infection (Buitenhuis, Rontved et al. 2011). The development of the innate immune response within young calves also suggests a malleable system within an individual that is susceptible to control via a mechanism other than genotype. My dissertation research studies aimed to contribute knowledge to several specific areas within the field of bovine mastitis and epigenetics. First, to describe the variation in recognition and response to LPS both across a sample population, as well as within an individual over time, dermal fibroblasts were collected and exposed to agonists eliciting an immune response which correlated with *in vivo* challenges on the animal. Secondly, a subset of these dermal fibroblasts had their methylomes mapped using MIRA-Seq technology in order to add to the currently sparse encyclopedia of tissues with epigenetic mapping publicly available. Finally, a comparison of immune response within cells was conducted against genome-wide methylation levels in order to determine regions of the genome susceptible to DNA methylation that may have an effect on innate immunity. This work will add to the current understanding of the innate immune response as well as potential mechanisms of control that have not previously been described in the bovine system.

CHAPTER 2:

The use of dermal fibroblasts as a predictive tool of the TLR4 response pathway and its development in Holstein heifers.

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2.1 Abstract

The innate immune system comprises the host's first line of defense against invading pathogens, and variation in the magnitude of this response between animals has been shown to affect susceptibility to mastitis. The toll-like receptor (TLR) family of proteins initiates the response to invading bacteria, specifically with TLR4 recognizing lipopolysaccharide (LPS) of Gram-negative microbes. The underlying genetic variation in the TLR4 pathway leading to differential response is not well understood; therefore, the objective of this work was to determine the efficacy in which the response to LPS by dermal fibroblasts could be used to predict the actual systemic response of that animal to an intravenous endotoxin challenge. To accomplish this, dermal fibroblasts were isolated from 15 Holstein heifers at 5, 11, and 16 months of age, exposed to either LPS or IL-1 β , and the production of IL-8 in media quantified by ELISA. Animals were ranked based upon the magnitude of the fibroblast IL-8 response and 8 heifers were selected (4 low-, 4 high-responsive) for challenge with an intravenous bolus dose (0.5 μ g/kg BW) of LPS. Overall between-animal variation in fibroblast IL-8 production following LPS or IL-1 β was high, indicating appreciable differences in the TLR4 pathway of the animals. Ranking of the fibroblast responses was consistent across the three sampling times for each animal, however the absolute response increased with age at which the fibroblasts were obtained consistent with the potential for age-related changes in cell function to impact immune function processes. Following systemic LPS challenge, heifers selected as high responders (HR) had higher plasma concentrations of TNF- α and IL-8 than heifers selected as low responders (LR).

LR heifers had a stronger febrile response than HR however. The use of dermal fibroblasts under laboratory settings appears to represent a practical model for predicting the innate immune response *in vivo*, and could act as an important tool in mapping genetic differences of the TLR4 pathway.

2.2 Introduction

Mastitis is considered the most costly production-related disease within the dairy industry due to a decrease in milk yield and quality (Schukken, Hertl et al. 2009), a decrease in lactation persistency (Capuco, Ellis et al. 2003), lowered fertility rates (Hansen, Soto et al. 2004), and an increase in pre-mature culling of infected animals (Seegers, Fourichon et al. 2003). Both Gram-positive and Gram-negative bacteria can infiltrate through the teat canal leading to mammary gland inflammation. Severity of infections caused by Gram-negative bacteria, such as *Escherichia coli*, is quite variable between cows suggesting an effect of individual host factors (Burvenich, Merris et al. 2003). The innate immune response represents the first line of defense against pathogens, and is a likely candidate for genetic polymorphisms responsible for the high degree of variability seen in mastitis susceptibility (Boulanger, Bureau et al. 2003).

The toll-like receptor (TLR) family of proteins recognize conserved bacterial motifs known as pathogen associated molecular patterns (PAMP), which lead to the induction of the innate immune response (Kopp and Medzhitov 2003). Each TLR is capable of recognizing different PAMP, with TLR4 recognizing LPS of Gram-negative bacteria. In response to TLR4 stimulation, an intracellular signaling pathway leads to

the eventual activation of the transcription factor NF- κ B, which has shown increased expression in the milk cells of infected cows (Boulanger, Bureau et al. 2003).

Genetic polymorphisms within genes of the TLR4 signaling pathway have been shown to have a major impact on the host response to LPS, specifically in the production of cytokines (Davidson, Currie et al. 2006, Rallabhandi, Awomoyi et al. 2008). Data have shown a correlation between cytokine production and NF- κ B activity, suggesting a link between these polymorphisms and transcription factor activity. As well, to highlight the impact of genetic variation, polymorphisms within the *CXCR1* receptor gene have also been linked to differing susceptibility to infection and neutrophil function in relation to clinical mastitis (Youngerman, Saxton et al. 2004, Pighetti and Rambeaud 2006).

Understanding the relationship of genetic make-up to mastitis susceptibility would represent an important selection tool for farmers to increase profitability of cows. The ability to predict a cow's likelihood of contracting an infection, and the subsequent response, have been difficult to track however under *in vivo* conditions due to numerous environmental factors associated with disease risk. Work has shown that both stage of lactation (Hogan, Smith et al. 1989, De Schepper, De Ketelaere et al. 2008), management practices (Goodhope and Meek 1980), and previous mastitis history (Houben, Dijkhuizen et al. 1993, Hertl, Grohn et al. 2010), impact susceptibility to mastitis. Due to this, it is important to establish a model that controls these factors while investigating an animal's innate immune response capability.

Human fibroblasts have been shown to generate a robust response to LPS exposure under laboratory conditions (Davidson, Currie et al. 2006, von Bernuth, Picard et al. 2008). The elimination of environmental factors, along with the ability to cryopreserve the cells while maintaining consistent responses to LPS, makes them an ideal model to investigate inter-animal variation in innate immunity. This is in contrast with cells collected from bovine milk, which have recently been reported to be unresponsive to LPS (Baumert, Bruckmaier et al. 2009). Also, the challenge of blood or blood derived cells has been unable to link the magnitude of response *in vitro* with the response of the same individuals (Bakiyeva, Brooks et al. 2005) or animals under *in vivo* investigation (Elsasser, Blum et al. 2005) due to high individual variation. While some studies have been able to link neutrophil activity with mastitis clearance (Heyneman, Burvenich et al. 1990, Lohuis, Schukken et al. 1990) and recently reviewed (Burvenich, Bannerman et al. 2007), fibroblasts offer a cellular model that can be used to define phenotypic values more closely aligned to TLR4 signaling, offering insight into not only mastitis clearance, but general mechanisms of disease response. Due to the major role of TLR4 in innate immune signaling and its relation to mastitis susceptibility, it represents an important keystone for understanding disease resistance in dairy cows.

An acceptable *in vitro* model to monitor LPS responsiveness would contribute to further investigation of the molecular underpinnings of mastitis vulnerability. We hypothesized that cultured dermal fibroblasts, isolated from Holstein heifers would show a high degree of between-animal variability in response to LPS challenge, with

the magnitude of the *in vitro* response being predictive of the animal's response to an intravenous LPS challenge. Key to this hypothesis is the consistency of response in samples obtained throughout the heifers' maturation.

2.3 Materials and Methods

2.3.1 Animals and Experimental Design

All experiments were performed in accordance with approval of the Institutional Animal Care and Use Committee at the University of Vermont. Fifteen Holstein heifers were randomly selected for use in the experiment. The animals were selected from a single herd raised at the University of Vermont Miller Research Farm. During the study period, heifers were fed the same ration and were housed in the same facility.

Fibroblast cultures were established from skin biopsies that were collected when the heifers were aged 5, 11, and 16 months (165 ± 30 d, 337 ± 12 d, and 487 ± 22 d, respectively). Following analysis of between-animal variation in the fibroblast production of IL-8 in response to LPS for the first two biopsy times, the four lowest and four highest heifers were selected for an *in vivo* LPS challenge. The final skin biopsy was obtained from all 15 animals approximately three months (87 ± 7 d) after the *in vivo* LPS challenge.

2.3.2 Fibroblast Collection and Isolation

A small skin sample from the shoulder region was obtained from animals that had been sedated using xylazine (0.03 mg/kg) injected intravenously through the tail vein. The surgical area was shaved, then cleansed with alternating washes of 70%

ethanol and betadine, and then anaesthetized using 10 mL of 2% Lidocaine. Two elliptical incisions were made superficial to the trapezius muscle forming an area of approximately 4 cm². Excised skin was placed into sterile PBS (Invitrogen, Grand Island, NY) containing 1X penicillin/streptomycin/Fungizone (100 units/mL, 100 µg/mL, and 0.25 µg/mL, respectively, Thermo Scientific, Rockford, IL) and transported to the laboratory on ice.

2.3.3 Fibroblast Primary Culture

Tissue samples were cut into mm³ size pieces by opposing scalpel blades to increase surface area, and then washed 3X in PBS by vigorous shaking. Tissue was finally incubated in 0.05% Type-I collagenase (MP Biomedicals, Solon, OH) diluted in plain Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) for 6 h with orbital shaking (200 rpm) at 37°C. Following collagenase digestion, the resulting cells were cultured in DMEM with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO), 1% insulin-transferrin-selenium (ITS; Invitrogen), and 1X penicillin/streptomycin/Fungizone in a T-25cm² flask at 37°C with 5% CO₂. After being grown to confluence (~4 days), cells were lifted with 0.25% Trypsin-EDTA (Thermo-Scientific) and transferred to a T-75cm² flask with similar media except for 5% FBS. Cells were expanded in three T-75cm² and finally aliquoted in DMEM with 20% FBS and 10% DMSO for storage in liquid nitrogen.

2.3.4 Fibroblast Challenge with LPS and IL-1β

Following recovery from cryopreservation and expansion in a T-75cm² flask to 70% confluency, cells were trypsinized and seeded in 6-well culture plates at 1.25×10^5

cells/mL in a total volume of 2 mL. After a 48-h incubation under normal growth conditions, cells were then exposed to either control or treatment media. Treatment consisted of growth media supplemented with either 100 ng/mL of ultra-pure LPS isolated from *Escherichia coli* O111:B4 (Sigma-Aldrich) or 1 ng/mL of recombinant bovine IL-1 β (AbD Serotec, Raleigh, NC). Treatment exposure to either LPS or IL-1 β lasted for 36 or 24 h, respectively. Following appropriate exposure time, media was removed, spun at 1500 r.p.m. to remove cell debris, and immediately stored at -20°C until further analysis, which occurred within several days of freezing. Cells were challenged in two groups, composed of samples from either 7 or 8 heifers, each treated under the same conditions. Grouping showed no effect on response to LPS or IL-1 β , and was not analyzed further. Experiments were replicated three times for biopsy one, and two times for biopsies two and three.

2.3.5 Quantification of IL-8

Production of IL-8 in response to either LPS or IL-1 β stimulation was quantified by custom bovine IL-8 ELISA. Briefly, wells were coated with 100 μ L of a 6.9 μ g/mL solution of mouse anti-bovine recombinant IL-8 monoclonal antibody (clone 170.13 generously gifted by Dr. Samuel Maheswaren (Malazdrewich, Ames et al. 2001)) diluted in a 0.05M bi-carbonate buffer and incubated overnight at 4°C. The plate was washed 3X with 0.05% PBS-Tween 20 and media samples were plated along with known standards made up of recombinant bovine IL-8 (Thermo Scientific) for 2 h. Again, the plate was washed, followed by the addition of 0.05 μ g/mL biotinylated goat anti-human mAb CXCL8/IL-8 (R&D System) again for 2 h. Following this 0.5 μ g/mL

Streptavidin-HRP (Sigma-Aldrich) was plated and incubated for 30 min. Finally, TMB substrate (Sigma-Aldrich) was added for approximately 5 min, with the reaction stopped by the addition of 1M sulfuric acid (H₂SO₄). Plate reactivity was quantified by measuring absorbance at 450nm and analyzed by a four-parameter analysis with OD corrected against blank wells (Synergy-HT; Bio-Tek, Winooski, VT).

2.3.6 *in vivo* Endotoxin Challenge

Heifers were selected for an *in vivo* challenge to take place between the second and third skin biopsies based upon their fibroblast's IL-8 response to LPS from the first two *in vitro* challenges. Animals were selected based upon their *in vitro* IL-8 response deviating greater than one standard deviation from the mean. This however produced three animals per group, which we considered too small a sample size and therefore supplemented with the next highest or lowest responder yielding n=4/group. The resulting animals therefore deviated at a minimum of either less than 0.8 SD or more than 0.75 SD from the group mean (n=15).

Animals chosen for the systemic LPS challenge were housed in single capacity stalls with free access to feed and water for 24 hours prior to challenge. During this acclimation period, Teflon cannula (Jorgenson Labs, Loveland, CO) were installed in the jugular veins of the animals with void space filled by heparinized saline to prevent clotting. Heifers were given a bolus dose of 0.5µg/kg BW LPS isolated from *Escherichia coli* O111:B4 (Sigma-Aldrich) at hour 0 via jugular catheters. Heparinized blood samples were taken at -30 min, and then hourly for 8 h. Plasma samples were

stored at -20°C. As well, the febrile response of the animals was monitored via rectal thermometer.

2.3.7 Plasma Protein Quantification

Plasma concentrations of bovine IL-6 were quantified by commercially available ELISA (Thermo-Scientific) following manufacturer's instructions. TNF- α concentrations were measured via RIA as previously described (Elsasser, Blum et al. 2005). Plasma cortisol concentrations were quantified by commercial ELISA (VWR, West Chester, PA) under manufacturer's directions. SAA was measured by ELISA as previously described (Kahl and Elsasser 2006). IL-8 was quantified by the previously described ELISA technique.

2.3.8 Quantitative Real-time PCR

Total RNA was prepared using an RNeasy Mini Kit (Qiagen, Valencia, CA) from fibroblasts of the 8 heifers previously selected for the *in vivo* experiment. Cells were recovered from cryopreservation, expanded and seeded into 6-well plates. At approximately 70% confluency the cells were then either kept under control conditions or treated with LPS (100 ng/mL) for 8 h. Following extraction, RNA quality was assessed for protein and salt contamination. First strand cDNA synthesis was conducted using the Improm II Reverse Transcriptase Kit (Promega). Messenger RNA expression was quantified by real time PCR with a 7500 Fastrun Machine (Applied Biosystems) using PerfeCTa SYBR Green Super-Mix, Low ROX kit (Quanta Biosciences). Messenger RNA expression for target genes was quantified against the endogenous control β -actin to normalize data. Messenger RNA was detected using synthesized

oligonucleotide primers as follows (Pareek, Wellnitz et al. 2005, Ibeagha-Awemu, Lee et al. 2007): *IL-8*: 5'- GCTGGCTGTTGCTCTCTTG-3' for forward primer; 5'-AGGTGTGGAATGTGTTTTTATGC-3' for reverse primer; *TLR4*: 5'-ACTGCAGCTTCAACCGTATC-3' for forward primer; 5'-TAAAGGCTCTGCACACATCA -3' for reverse primer; and β -actin (*ACTB*): 5'-GCAAATGCTTCTAGGCGGACT-3' for forward primer; 5'-CAATCTCATCTCGTTTTCTGC G-3' for reverse primer.

2.3.9 Statistical Analysis

The correlation of relative ranking of IL-8 and IL-1 β production between sampling times was determined by Spearman's rank correlation coefficient (r_s) using the Prism 5.0 software package (GraphPad Software Inc., La Jolla, CA). Age dependent response of fibroblasts to treatment was analyzed by repeated measure one-way ANOVA with Tukey's means separation test (Prism 5.0). Cytokine responses to the *in vivo* LPS challenge were analyzed by comparison of area under the curve (AUC) using trapezoidal summation (Prism, 5.0) for each time point and concentration. AUC was compared for grouping differences using a two-tailed Student's t-test. mRNA expression levels were analyzed by PROC Mixed (SAS 9.2, Cary, NC) with covariance to account for repeated measures of both time and treatment. Comparisons with $P < 0.05$ were considered statistically significant within experiments.

2.4 Results

2.4.1 Dermal Fibroblast Response to LPS and IL-1 β

IL-8 was undetectable (minimum detectable limit=156 pg/mL) in fibroblast conditioned media from control wells while its production in response to both LPS and IL-1 β was highly variable between heifers throughout all three sampling points (**Figure 1**). Following 36 h exposure to LPS (100 ng/ml), the highest responsive fibroblast cultures produced approximately 3.5 and 3.0 fold more IL-8 than the lowest responsive cultures from the second and third biopsies, respectively. Relation of response could not be calculated for the first biopsy due to the unresponsiveness of two samples to LPS. Across all three biopsies the animals maintained a consistent ranking relative to one another in terms of IL-8 production in response to LPS. This was confirmed by determining the Spearman's rank correlation coefficient that gave r_s values of 0.77, 0.88, and 0.90 for comparisons of biopsy one to two, one to three, and two to three, respectively. All of these values were significant ($P < 0.001$).

In response to 24 h exposure to IL-1 β (1 ng/ml), the cultures showed a similar between-animal variation in IL-8 production. The highest responsive cultures produced 9.5, 4.5, and 2.25 fold more IL-8 in response to IL-1 β from the first, second, and third biopsy collections, respectively, as compared to the lowest responding cultures (**Figure 1**). Again, the heifers maintained a relative consistency across the three biopsies in the rank of their fibroblast IL-8 response relative to others within the study. Analysis using Spearman's rank correlation produced r_s values of 0.47, 0.62, and 0.49 for comparisons

of biopsy one to two, one to three, and two to three, respectively. These comparisons approached significance ($0.05 < P < 0.10$).

Fibroblasts from each animal were treated with LPS and IL-1 β at the same time under the same conditions except that media was harvested following 36 h or 24 h, respectively. Even with the reduced exposure time, the magnitude of IL-8 production in response to IL-1 β (1 ng/ml) was much greater than that in response to LPS (100 ng/ml). Due to sharing of the myeloid differentiation primary response gene (88) (MyD88)-dependent response pathways within the cell, a correlated production of IL-8 to the two ligands was expected. Correlation analysis indicated a moderate positive correlation throughout all three sampling time points (**Figure 2**) giving R^2 values of 0.30, 0.43, and 0.66 respectively. This was confirmed by Spearman's rank correlation coefficient on relative rank of response to LPS and IL-1 β within a biopsy. Analysis indicated similar ranks ($P < 0.05$) for all three biopsies when comparing response to the two ligands.

2.4.2 Dermal Fibroblast Production of IL-6 in Response to LPS

To confirm the variation of response to LPS as being due to differences within the TLR4 signaling pathway and not as a result of variation within the *IL-8* gene alone, media from the third sampling challenge was analyzed for IL-6 production. Production of IL-6 in response to LPS showed a moderately strong correlation to IL-8 production (**Figure 2D**) with an $R^2 = 0.67$, significant at $P < 0.05$. Individual ranking based upon the two measurement parameters was consistent as well, with heifers occupying a low or high rank whether analyzed for IL-6 or IL-8 production.

2.4.3 Age-Dependent Development of Fibroblast Responsiveness to LPS and IL-1 β

Responsiveness to LPS within the fibroblast model over developmental age was monitored to determine the stability of the response. (**Figure 3A**). The age at which fibroblast samples were taken was shown to have an effect ($P < 0.01$) on IL-8 production. IL-8 production in response to LPS increased over each sampling time, with production highest at the 16-month time point. It should be noted that for the sampling at 16 months of age, fourteen of the fifteen heifers were verified pregnant. The relative ranking of response for the third sampling of the unbred heifer (denoted in Figure 1 as heifer number 4) did not appear to have been affected by the difference. Although IL-8 production increased with age, the relative rank of IL-8 production in response to LPS between animals was consistent over all time points. This developmental increase in IL-8 production was also seen in response to fibroblast stimulation with IL-1 β (**Figure 3B**). Again, age at sampling time had an effect ($P < 0.01$), showing a distinct increase in IL-8 production in fibroblasts' response to IL-1 β from older heifers.

2.4.4 TLR4 and IL-8 mRNA Expression in LR and HR Fibroblasts

Following initial experiments to determine IL-8 protein production in response to LPS, cells from the four highest and four lowest fibroblast cultures were recovered from cryopreservation and re-challenged with LPS for 8 h to provide total mRNA for molecular analysis. *IL-8* mRNA in control fibroblasts was only detectable at very low levels ($C_t \approx 35$ cycles) (**Figure 4A**). A marked induction was observed following LPS treatment, with HR fibroblasts expressing higher ($P < 0.05$) levels of IL-8 than LR

fibroblasts overall. Following LPS treatment HR fibroblasts had a 9.1, 2.9, and 3.5 fold higher expression of IL-8 than similarly treated LR cells from the first, second, and third samplings, respectively. Expression of *IL-8* also increased as a result of age ($P < 0.01$) confirming the increase of IL-8 production detected in media.

The cellular response to LPS is mediated via the TLR4 receptor and thus its expression was an important factor to consider in causing the differential responses in IL-8 induction. LPS exposure did not lead to an induction ($p=0.64$) of *TLR4* expression in any fibroblast cultures. *TLR4* expression levels also showed no difference ($p=0.32$) between LR and HR fibroblasts (**Figure 4B**). One difference seen though, was the effect of age ($P < 0.01$) on *TLR4* expression, with a detectable decrease in gene transcript levels with maturation.

2.4.5 Systemic Response to Intravenous LPS Administration

Heifers selected as the four lowest (LR) and four highest (HR) responders were given an intravenous dose of LPS and then monitored for plasma cytokine levels as well as body temperature. Plasma levels of IL-8 were undetectable at the time of LPS administration but then increased markedly within one hour (**Figure 5A**). HR and LR IL-8 levels both displayed an acute increase within two hours post-LPS exposure. At this time point, HR concentrations (6.43 ± 0.62 ng/mL) were higher ($P < 0.05$) than LR levels (4.16 ± 0.32 ng/mL). As well as showing higher peak IL-8 levels, the overall profile of IL-8 concentration over the 8 h measurement period was ($P < 0.05$) higher in HR than in LR heifers.

Plasma levels of TNF- α at the time of LPS administration (time=0) were not different between heifers grouped as LR or HR animals (**Figure 5B**). All animals responded to LPS with a marked production of TNF- α within one hour that reached maximum concentration between one and two hours following LPS administration. HR TNF- α concentrations were higher at their peak (time = 2; TNF- α = 3.16 ± 0.66 ng/mL) than LR peak concentrations (time = 1; TNF- α = 1.95 ± 0.05 ng/mL). Both LR and HR TNF- α levels slowly decreased until reaching approximate basal levels at the final measurement (time= 8). Investigating the overall response to LPS over the eight-hour period of measurement, HR animals showed a higher ($P < 0.05$) TNF- α concentration profile than LR animals.

Plasma concentrations of cortisol were also measured to monitor the stress response of the heifers to the endotoxin challenge. Cortisol concentrations increased within one hour of LPS administration, rising until highest concentrations were reached at hour 3 for both LR (48.5 ± 5.2 ng/mL) and HR (57.2 ± 6.6 ng/mL) (**Figure 5C**). While the average cortisol concentration was higher for HR from hours 1-7, the differences were not statistically significant ($P = 0.15$) between the two groups. Cortisol concentrations dropped dramatically by hour 7 to levels slightly above concentrations seen at hour 0.

Levels of IL-6 following LPS administration developed with a similar profile to IL-8, beginning at an undetectable level, followed by a rapid increase. IL-6 levels reached maximal levels at hour 4 in LR heifers (4.32 ± 0.75 ng/mL), while not reaching a maximum concentration in HR heifers until hour 6 (3.38 ± 0.21 ng/mL) (**Figure 5D**).

There was no significant difference ($P = 0.46$) seen in IL-6 production following LPS challenge between the two groups.

SAA levels were monitored infrequently and within the 8-hour time frame and the complete profile of the response was not captured due to the lag response typical for this protein (**Figure 5E**). After 8 h the concentration of SAA was still increasing in all animals compared to the prior time point. In this, the full resolution of the response was not captured, and no significant difference was seen between the two groups.

Rectal temperature was measured and revealed a marked febrile response to LPS administration. (**Figure 5F**). Temperature increased slowly, not reaching the highest point until hours 5 and 6 for the LR ($39.7 \pm 0.16^\circ\text{C}$) and HR ($38.8 \pm 0.13^\circ\text{C}$) heifers, respectively. Overall, when examining the temperature of the heifers over the period of measurement, LR showed a higher ($P < 0.05$) temperature than HR.

2.5 Discussion

The economic effects of mastitis are unquestionable, with infections related to lowered lactation persistency (Capuco, Ellis et al. 2003) and a continually decreasing milk output following repeated cases (Schukken, Hertl et al. 2009). Considerable variation in mediators of innate immunity has been seen between animals in response to intramammary challenge with *E. coli* (Bannerman, Kauf et al. 2008) or LPS (Jacobsen, Toelboell et al. 2005) likely due to underlying genetic differences between animals. The TLR4 pathway, the first line of defense against *E. coli* invasion, represents a promising area of investigation to understand the basis of this variation.

The goal of the present study was to determine the effectiveness of the use of dermal fibroblasts in laboratory conditions to predict the response to an intravenous LPS challenge in an animal model, as well as to monitor the stability of the measured response in cells taken from heifers over their maturation. Establishing an effective model of response to LPS allows for the investigation of underlying mechanisms of differential TLR4 pathway activation. As well, understanding the relationship of the immune response with age can help to determine the consistency of the predictive model and determine the threshold age at which the immune response can be confidently analyzed. Based upon analysis conducted within this study, the relative immune response of an animal may be investigated as early as 5 months of age. An important distinction however is that for this to occur, age between individuals should be matched, as our results show a response that is not absolute and increases at least until 16 months of age. In this, comparing heifers of varying age is not an effective tool.

In the future, directed breeding programs may likely be used with more specific genetic-based strategies in mind. Selection tools have been shown to reduce mastitis incidence in offspring (Heringstad, Klemetsdal et al. 2003, Heringstad, Klemetsdal et al. 2007), however a better understanding of the genetic polymorphisms underlying the immunological response would play a major role in improving selection techniques. A hurdle in current selection strategies is the accurate identification of a desired phenotype, in which an animal quickly mounts an adequate response to clear a bacterial invasion with limited damage to self. Assigning superiority to either high or low-responsiveness is difficult due to the contradictory requirements of a strong response

that will clear infection, without the over production of cytokines that may lead to systemic disease and an increased mortality in the host (Takeda and Akira 2005). By identifying specific candidate genes playing a role in the differential production on cytokines, an even more specific approach may be taken to reducing mastitis incidence.

The random selection of 15 heifers from which to isolate fibroblasts revealed a large range of IL-8 produced in response to LPS exposure. This was to be expected even within our small sample, as previous work has cited a high degree of between-animal variability in the measurement of immune markers following both *in vitro* and *in vivo* exposure to TLR4 ligands (Elsasser, Blum et al. 2005, You, Karrow et al. 2008, Kahl, Elsasser et al. 2009). Importantly, within our model system, three separate biopsies taken from the 15 animals showed consistency in the between-animal variation. The ranking of the animals from low to high, based upon the LPS responsiveness of cells from the first sampling remained steady with the second and third samplings. This consistency of rank showed that the isolation of fibroblasts did not act as a variable between biopsies, and that over time the relative inter-animal ability of the cells to respond to LPS did not change. As well, the consistency of ranking seen in the 16-month sample indicates that the administration of LPS to eight of the fifteen heifers between the second and third biopsies during the *in vivo* trial did not affect the fibroblast response. The differences seen between LR and HR fibroblasts were also confirmed through mRNA expression levels of the IL-8 gene, with substantially higher message abundance in HR cells over all three samples following LPS treatment.

The fibroblasts also showed a positive correlation between their response to LPS and the response to IL-1 β . While the correlation between the responses increased across sampling times, this is likely due to the lower cytokine production (two unresponsive cows) in response to LPS within the five-month sampling period. This is highlighted by a consistent r_s value from the Spearman's analysis investigating relative rank of response to LPS and IL-1 β within a sampling time. The TLR4 (LPS) and IL-1R (IL-1 β) receptors share downstream signaling proteins, which lead to the eventual up-regulation of NF- κ B responsive genes (Verstrepen, Bekaert et al. 2008). There are however slight variations within the signaling cascade, which make the comparative response to the two ligands an important diagnostic tool in identifying possible genetic defects of signaling. For example, in contrast to IL-1R, signaling through the TLR4 receptor requires accessory proteins such as cluster of differentiation 14 (CD14), lipopolysaccharide binding protein (LBP), and lymphocyte antigen 96 (MD-2). As well, TLR4 signaling is dependent upon a TIR-domain-containing adapter protein (TIRAP) as shown by TIRAP deficient mice, unable to respond to LPS stimulation (Horng, Barton et al. 2002, Yamamoto, Sato et al. 2002). Secondly, while IL-1R signals exclusively through recruitment of MyD88, the TLR4 pathway contains a MyD88-independent pathway (Verstrepen, Bekaert et al. 2008) capable of a stunted, but measurable NF- κ B activation (Kawai, Adachi et al. 1999). These differences, among others, allow for the comparative response to LPS and IL-1 β by the fibroblasts to be used to tease out target proteins for future genetic investigation. In the present study, the moderate correlation of response to LPS and IL-1 β suggests that polymorphisms

responsible for differences between the high and low responsive cultures are common to the two signaling pathways such as proteins involved in MyD88-dependent signaling.

While the fibroblast model showed little intra-animal variation in the ranking of HR and LR heifers at each specific age of sampling, and was predictive of the animal response to a systemic LPS challenge, absolute quantities of IL-8 produced *in vitro* in response to both LPS and IL-1 β were affected by age. Fibroblasts isolated from heifers aged 5 months produced lower amounts of IL-8 than those collected from the same heifers at the age of 11 or 16 months. This difference was confirmed through gene expression of the IL-8 gene in response to LPS.

Increased sensitivity to LPS with age has been described previously in a human monocyte model (Yan, Qing et al. 2004) in which levels of the adaptor protein MyD88 increased in correlation with age and LPS responsiveness. This model however was unable to describe the mechanistic action by which this change occurred. Several possible factors for the increased sensitivity to LPS with age have been described. One explanation suggests a requirement for a muted response in young animals during the establishment and proliferation of the gut microbiota. The lowered response to stimuli helps eliminate chronic fever and cytokine production to the presence of symbiotic bacteria (Zampetaki, Xiao et al. 2006, Foldes, Liu et al. 2010, Vamadevan, Fukata et al. 2010). This explanation involves mechanisms at the neonatal stage of development however, and would require a slow increase in the recognition of LPS to fall within the timeframe of our sampling. The gut response to microbes is weak when compared to

the response elicited by fibroblasts (Testro and Visvanathan 2009) which makes for a difficult comparison between the two. This developmental increase in responsiveness to LPS warrants further investigation in the ruminant system and into possible epigenetic control of the developing innate immune system.

Although we did not find a difference in *TLR4* expression between high and low responsive fibroblasts, possibly due to a low sample size, we did observe a consistent age-related decrease in its expression. Interestingly, as *TLR4* expression decreased, responsiveness to LPS as determined by IL-8 production increased. Following *TLR4* transcription there are several control points, which may also be playing a role in the differences seen between animals. *TLR4* is transiently shuttled between the endosome and cell surface by chaperone proteins, heat shock protein 90 (gp96) and protein associated with *TLR4* A (PRAT4A) until engaged by LPS (Saitoh 2009, McGettrick and O'Neill 2010). This offers an area of regulation not investigated within the current study, which may account for the age-related decrease in *TLR4* expression and subsequent increase in responsiveness to LPS.

The fibroblast expression of *TLR4* was not affected by exposure to LPS. Previous work has shown an induction of *TLR4* gene expression following a 24 h LPS treatment in a variety of cell types including bovine mammary MAC-T cells (Ibeagha-Awemu, Lee et al. 2007), bovine monocyte-derived macrophages (Taraktsoglou, Szalabska et al. 2011), and mammary tissue samples following experimental *E. coli* mastitis (Rinaldi, Li et al. 2010). However, others have shown that LPS has little effect on *TLR4* expression in bovine monocyte-derived macrophages (Franchini, Schweizer

et al. 2006) or even a substantial, albeit transient, down-regulation of *TLR4* following LPS challenge of murine macrophages (Nomura, Akashi et al. 2000). These studies represent work under both *in vitro* and *in vivo* conditions, across several cell types from cows, and in one case sheep. Our results, when compared to other studies suggest a possible tissue specific response pattern in the regulation of TLR4 expression.

Once IL-8 production had been measured from all heifer fibroblasts, the ranks that they had been assigned over the first two biopsies were averaged. The four lowest and four highest heifers were selected for intravenous LPS challenge to monitor systemic response to endotoxin. Administration of LPS led to an acute increase in all measured cytokines and a delayed increase in body temperature. HR heifers exhibited higher plasma concentrations of IL-8 and TNF- α in response to LPS in comparison with LR heifers. Production of these cytokines in response to LPS within our system is most likely to have originated from monocytes (Parameswaran and Patial 2010). IL-8, a chemo-attractant, plays an important role in the sequestration and activation of neutrophils into sites of infection such as the lungs of sheep or beef cattle (Malazdrewich, Ames et al. 2001, Herndon, Foreyt et al. 2010) or the mammary gland of dairy cows (Lahouassa, Moussay et al. 2007). TNF- α plays an important role in both inflammation and apoptosis following a major infection and hyperresponsiveness in TNF- α production has been shown to be a heritable trait (Elsasser, Blum et al. 2005). Some evidence suggests that infection outcome may be closely aligned with TNF- α levels, making it an important cytokine to monitor (Zanotti, Kumar et al. 2002, Cavaillon, Adib-conquy et al. 2003). This is most likely due to TNF- α acting as one of

the main mediators of endotoxin response, as illustrated through the reduction of LPS toxicity in mice with a TNF- α antigen blockade (Beutler, Milsark et al. 1985). Similar to IL-8, higher levels of TNF- α have not always been considered a productive response to infection as high levels of TNF- α have been shown to adversely lead to major organ failure (West and Heagy 2002).

The steroid marker cortisol was measured to determine the stress response of the heifers. The results were quite variable between animals although the response of the HR group was numerically higher. A high degree of variation in cortisol response to endotoxin challenge in beef cattle has been previously reported, with similar response profiles to our results (Carroll, Arthington et al. 2009). Signaling within the hypothalamic-pituitary-adrenal axis is an important factor determining cortisol levels following bacterial infection (Webster and Sternberg 2004), and has been shown to be the main variable controlling cortisol levels in sheep after endotoxin exposure (You, Karrow et al. 2008).

Following infection, the acute-phase response in animals plays an important role in pathogen resolution. Acute-phase proteins (APP) in blood contribute to restoring homeostasis and limiting microbial growth in an antibody-independent manner in animals following trauma. SAA is an APP linked to chemotaxis of monocytes, polymorphonuclear cells, and T cells, as well as down-regulating some inflammatory markers (Cray, Zaias et al. 2009). In our model, differences in SAA concentrations between HR and LR were not seen. This may be expected from SAA, which is

generally slow in activation and within our 8 hour time frame of measurement would not have reached full potential.

The febrile response of the heifers to the endotoxin challenge was characterized by the LR heifers developing a considerably higher rectal temperature, which was also slower to resolve. Interestingly, the febrile response to LPS in cattle is known to be quite variable between animals, and exhibits an inverse relationship with endotoxin dose (Jacobsen, Toelboell et al. 2005). These authors found that a low dose challenge with LPS (10 ng/kg) into mature cows caused a greater febrile response than doses of 100 or 1000 ng/kg. They also reported that the higher LPS doses caused greater elevations in inflammatory markers SAA and haptoglobin (Jacobsen, Andersen et al. 2004). Perhaps the LR heifers in the current study are analogous to cattle responding to a lower dose of LPS, and thus producing a greater febrile response in the face of a reduced inflammatory response.

The present study did not address the impact of a high versus low response to LPS on the actual outcome of bacterial infection such as mastitis or bacterial pneumonia. Studies with fibroblasts obtained from lactating cows and an experimental *E. coli* mastitis challenge are currently in progress in our laboratory. The predictability of the fibroblast model remains to be tested with additional PAMPs and pathogen challenge studies.

2.6 Conclusion

The results support the use of the isolated dermal fibroblasts under laboratory conditions as an effective model in mapping between-animal differences in the TLR4 response pathway. Previous attempts to use whole blood challenges from heifers proved to not be predictive of the overall responsiveness to LPS once the challenge was transferred to the animal model. In contrast, fibroblasts represent a promising venue for which to explore the underlying mechanisms leading to differential innate immune responses. The ability to predict animal responses to bacterial stimuli shows promise in developing breeding programs aimed towards the selection of animals having an appropriate response to infections. In doing so, this could lead to improved animal welfare and a lower dependence on the use of antibiotics and antimicrobial agents.

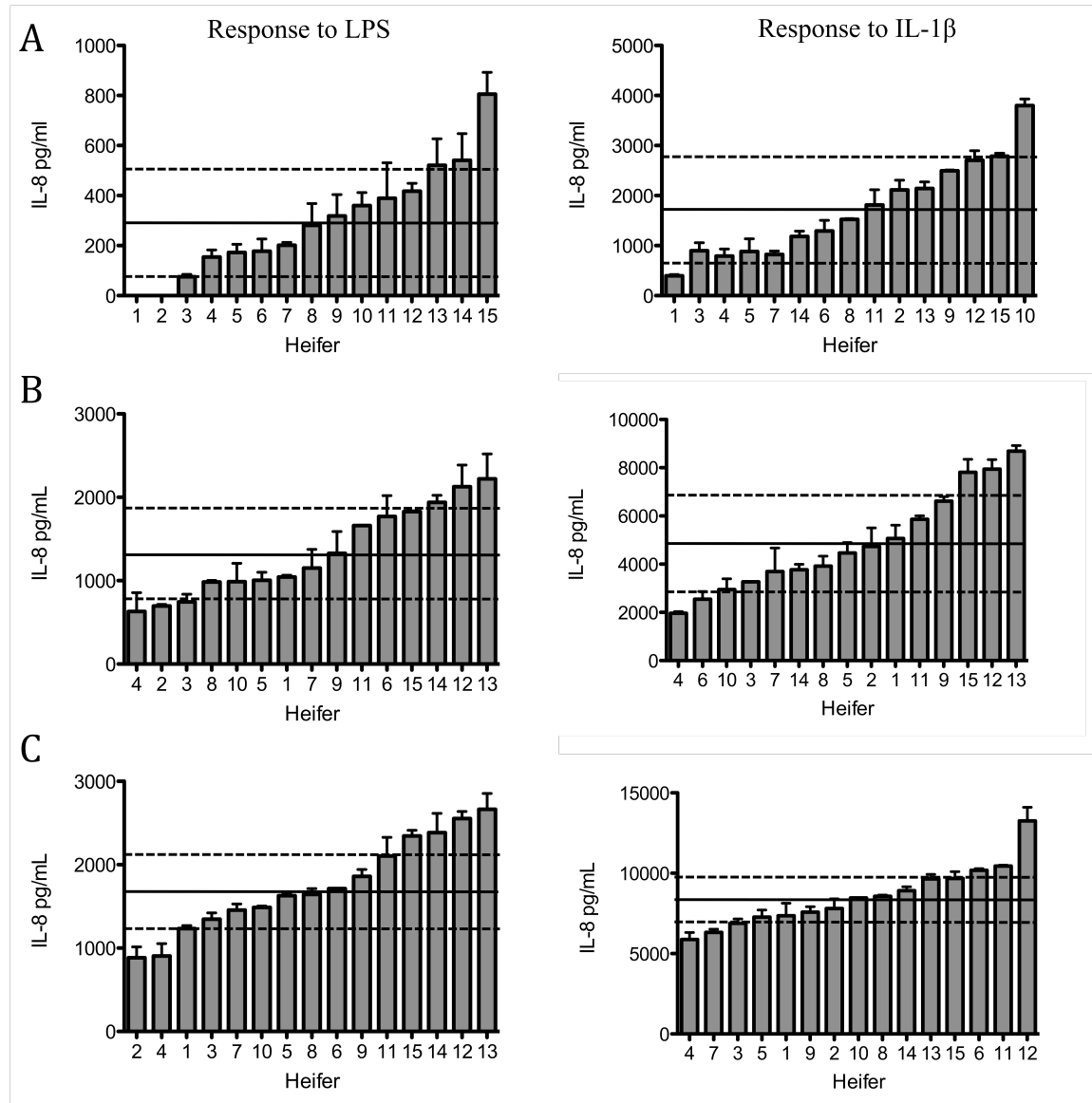


Figure 1. IL-8 production by cultured dermal fibroblasts in response to LPS (100 ng/ml) or IL-1 β (1 ng/ml) treatment for 36 and 24 hours, respectively. Fibroblasts were obtained from the same heifers at 5 months (**A**), at 11 months (**B**), or at 16 months of age (**C**). Fibroblasts exposed to media alone produced very little to undetectable levels of IL-8. Solid lines indicate group mean, dashed lines indicate plus/minus one S.D.

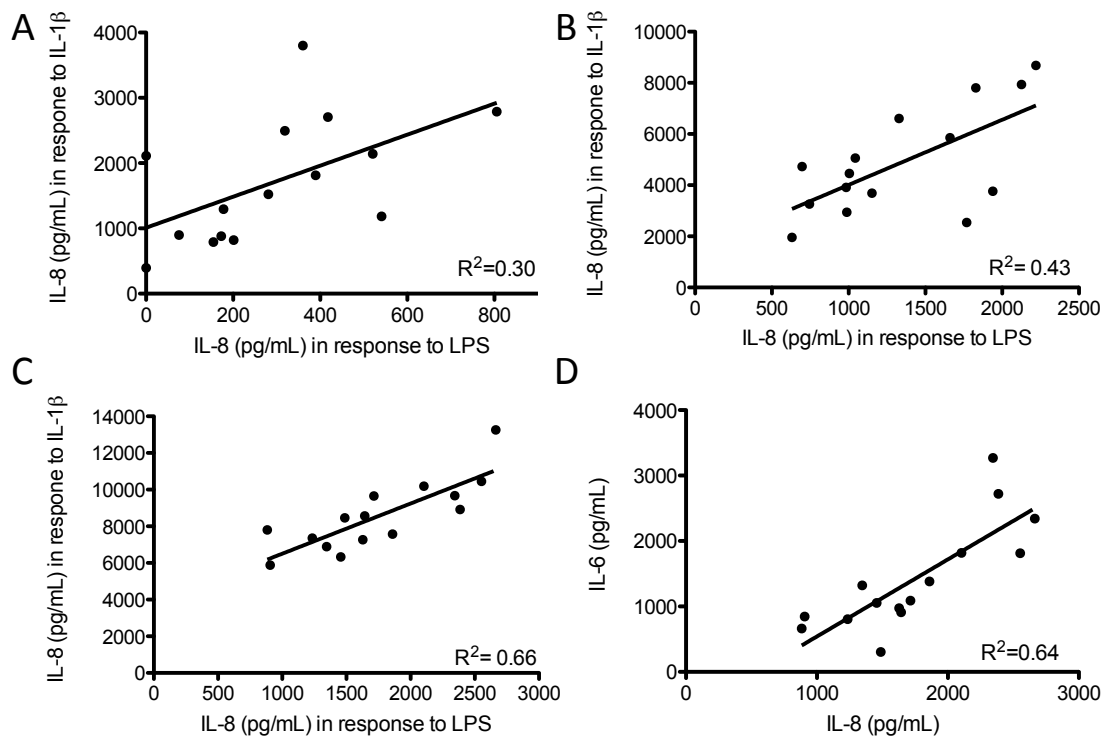


Figure 2. Correlation between IL-8 production by fibroblasts in response to LPS (100 ng/ml) or IL-1 β (1 ng/ml) treatment for 36 and 24 hours, respectively. Fibroblasts were obtained from the same heifers at age 5 months (**A**), at age 11 months (**B**), or at age 16 months (**C**). The relation of IL-8 and IL-6 production of fibroblasts isolated from heifers aged 16 months in response to LPS (**D**). Correlation coefficients depicted are significant ($P < 0.05$).

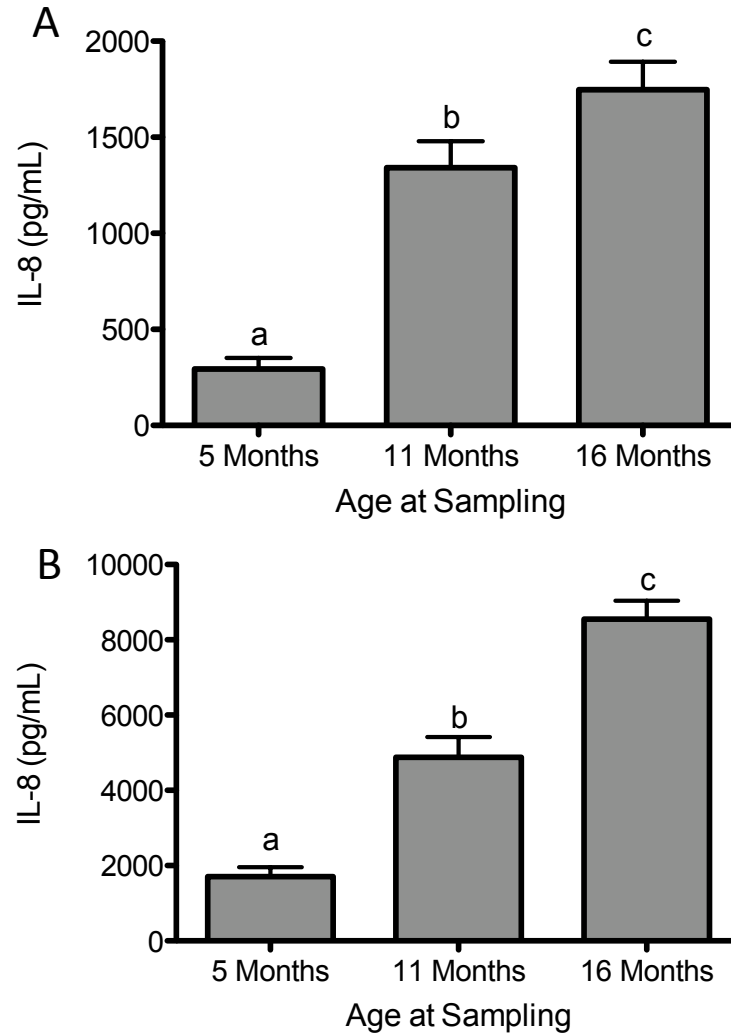


Figure 3. IL-8 production by fibroblasts in response to **(A)** LPS (100 ng/ml) or **(B)** IL-1 β (1 ng/ml) treatment for 36 and 24 hours, respectively. The fibroblast cultures were established from skin biopsies obtained from heifers at approximately 5, 11, and 16 months of age. Columns indicated with different letters differ ($P < 0.05$).

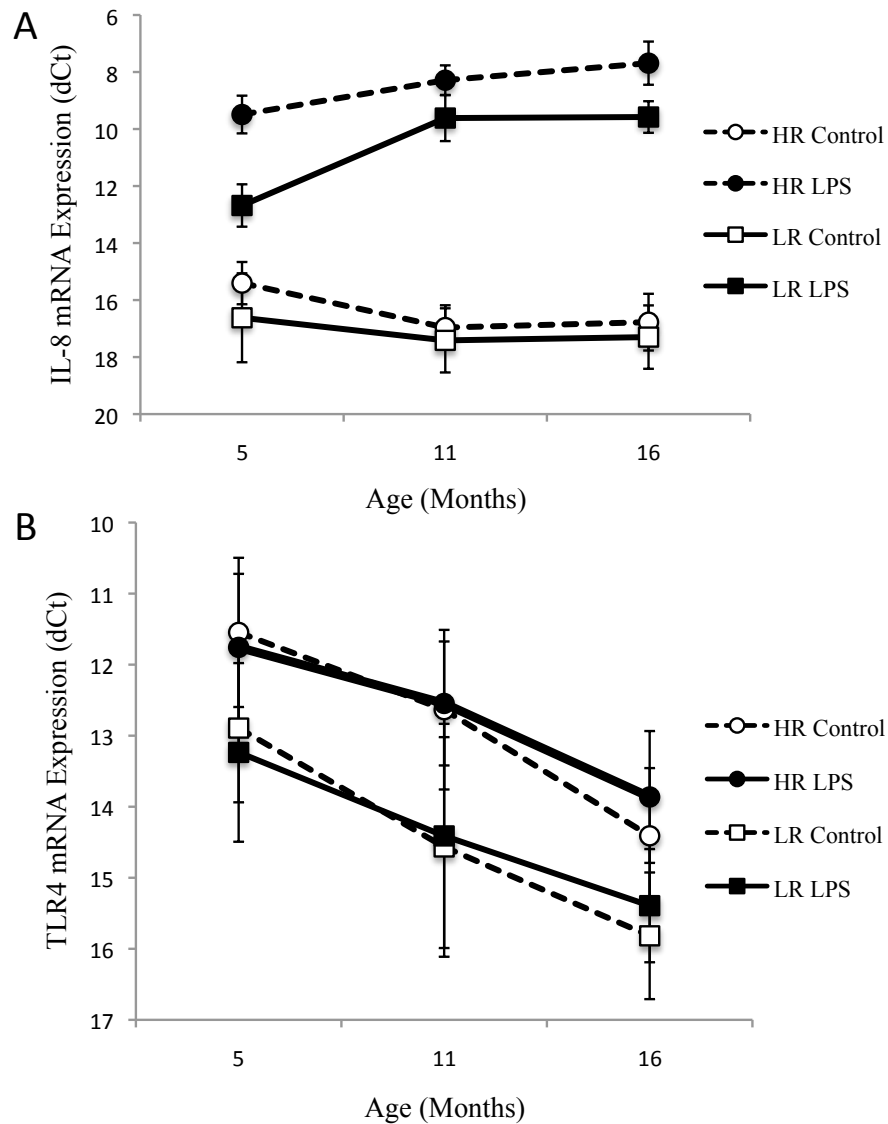


Figure 4. Expression of IL-8 mRNA (**A**) and TLR4 mRNA (**B**) by LR and HR fibroblasts under basal conditions and following exposure to LPS (100ng/ml; 8 h) as measured by RT-PCR. IL-8 expression was significantly higher ($P < 0.05$) in HR than LR cells under LPS stimulated conditions. Fibroblasts were established from skin biopsies obtained at 5, 11, 16 months of age. Values are expressed as dCt using β -actin expression as the endogenous control. All values are mean \pm S.E.M. (n=4/group).

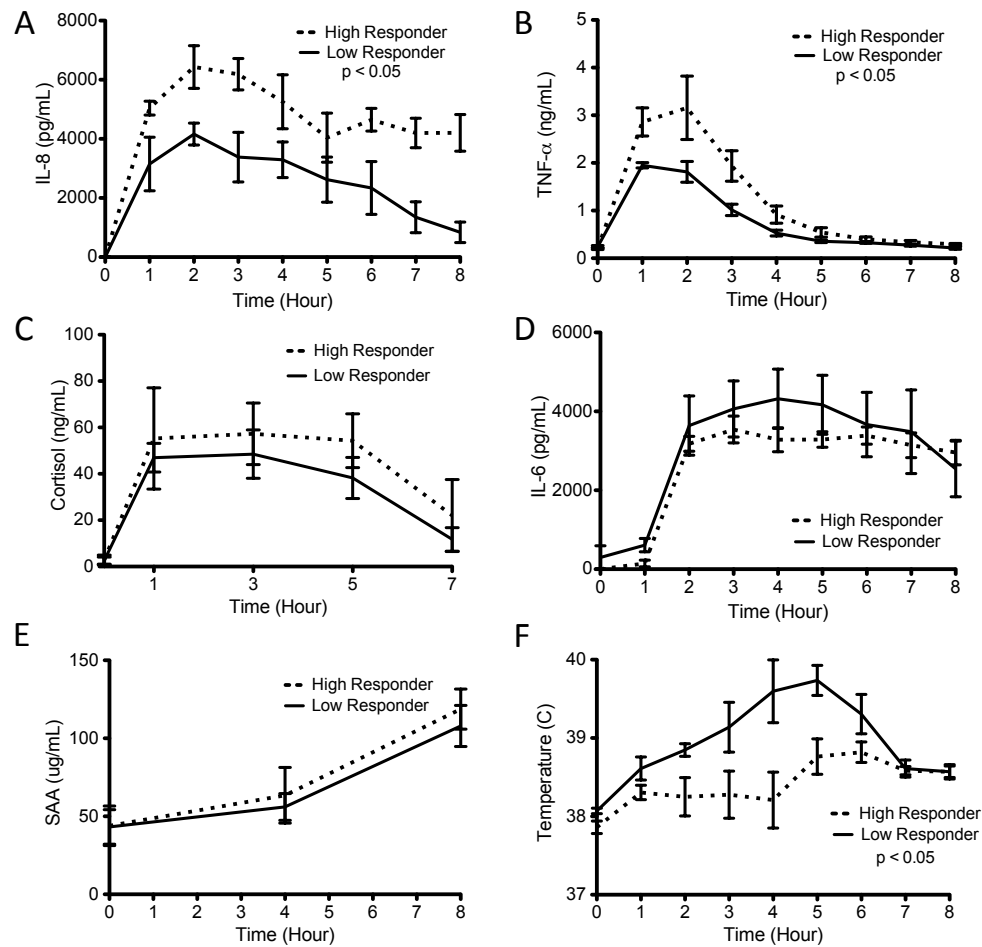


Figure 5. Profiles of immune related proteins in plasma, and rectal temperature of heifers challenged at 14 months of age with intravenous administration of 0.5 ug/kg BW LPS. Challenged animals were selected from the 15 animals depicted in Figure 1, and were the four lowest responders or four highest responders based on IL-8 production in response to LPS by their dermal fibroblasts obtained at 5 and 11 months of age. Parameters monitored for 8 hours following endotoxin challenge were plasma concentrations of (A) IL-8, (B) TNF- α , (C) cortisol, (D) IL-6, (E) SAA, and (F) rectal temperature. Data presented as mean \pm S.E.M. Differences between groups are indicated ($P < 0.05$).

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CHAPTER 3:

Epigenetic contribution to individual variation in response to lipopolysaccharide in bovine dermal fibroblasts.

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3.1 Abstract

The innate immune signaling pathway plays a crucial role in the recognition and early response to pathogens associated with disease. Genetic analysis has been unable to completely account for individual variability in the strength of the innate immune response. The aim of this study was to determine the role of the epigenetic markers (DNA methylation or histone acetylation) in controlling bovine gene expression in relation to the response to lipopolysaccharide (LPS). To determine the impact epigenetics may have in controlling innate immunity, dermal fibroblasts from fifteen dairy heifers having previously displayed a differential response to LPS were exposed to 5-aza-2'-deoxycytidine (AZA) and trichostatin A (TSA); de-methylating and hyper-acetylating agents, respectively. The AZA-TSA exposure resulted in a loss of variability between individuals' response to LPS as measured by fibroblast IL-8 protein production. Transcriptomic analysis by microarray was used to elucidate the role of epigenetics in innate immune signaling at 2, 4, and 8 hours post-LPS exposure. A subset of genes displayed altered expression due to AZA-TSA alone, suggesting an epigenetic regulatory element modifying expression under normal conditions. Treatment with AZA-TSA also led to increased expression of IL-8 (7.0 fold), IL-6 (2.5 fold), TNF- α (1.6 fold), and serum amyloid A 3 (SAA3) (11.3 fold) among other genes compared to control cultures for at least one of the measured times following LPS exposure. These data supports the conclusion that epigenetic regulation significantly alters LPS-induced responses and constitutive cytokine gene expression.

3.2 Introduction

There is a growing body of evidence to suggest that components of the adult immune response are established very early in life. The developmental origins of health and disease hypothesis suggests that environmental stimuli present following conception and until birth may play a role in increased rates of disease (Gluckman, Hanson et al. 2005). The fetal environment is closely associated with maternal status during pregnancy, and differences in maternal conditions have been associated with the development of diabetes, hypertension, and asthma in humans (Bousquet, Jacot et al. 2004, Gluckman, Hanson et al. 2005). Variation in the intrauterine environment throughout pregnancy may thus play a large role in determining the phenotype of the offspring. A proposed mechanism for environmental modulation of the immune response is through alteration of epigenetic markers important in controlling gene expression. Epigenetic effects are regulated through DNA methylation and histone acetylation that affect transcription factor access to DNA through chemical modification of DNA binding sites and alterations in chromatin structure, respectively (Bogdanović and Veenstra 2009, Sawan and Herceg 2010). For example, variation in methylation status of the *interleukin(IL)-6* and *IL-8* gene promoters in human cell models investigating periodontitis and rheumatoid arthritis appear to predispose some subjects to chronic inflammation through a hyper-responsiveness phenotype (Andia, de Oliveira et al. 2010, Ishida, Kobayashi et al. 2011). In addition to this, increasing histone acetylation in human intestinal epithelial cells (IEC) was able to potentiate the cellular response to lipopolysaccharide (LPS) as measured by IL-8 protein production

(Angrisano, Pero et al. 2010). Components of the pathogen detection and signaling pathways mediating the response to LPS have also been implicated as regions susceptible to epigenetic control. Epigenetic suppression, mediated by DNA-methylation, of *toll-like receptor (TLR)-4* gene expression has been linked to a lowered response to LPS in intestinal epithelial cells (Takahashi, Sugi et al. 2009). Conversely, DNA hypomethylation has been implicated in over expression of *myeloid differentiation factor (MD)-2* in human IECs leading to higher responsiveness to LPS exposure (Vamadevan, Fukata et al. 2010). These findings suggest that methylation and acetylation may play an important role in the regulation of immune-responsive genes involved in pathogen recognition and subsequent signaling. Investigation of the role of epigenetic variation between individuals in modifying their immune response capability would benefit our understanding of human and animal health.

Studies conducted on pregnant rats have shown that prenatal exposure to LPS leads to a suppressed innate immune response in offspring when examined at 5 days post birth (Hodyl, Krivanek et al. 2008) or even after 40 weeks of life (Williams, Teeling et al. 2011). Considering the ability of the maternal environment to influence the adult immune response (through epigenetic modulation), variation in the intrauterine environment may play a major role in causing individual variation in susceptibility to disease. The dairy cow is one animal for which maternal environment is not uncommonly associated with metabolic or infectious disease. A goal of dairy production is to ensure that dairy cows in their second or greater lactations are also pregnant. Mastitis and other systemic infections are not uncommon occurrences during

a dairy cow's pregnancy and may affect the phenotypic response to pathogens exhibited by her offspring. Interestingly, dairy cows exhibit a range of responses to experimental mastitis challenge and yet traits associated with mastitis such as milk somatic cell count and incidence of clinical mastitis have very low heritability (Dal Zotto, De Marchi et al. 2007), suggesting only a minor genetic influence. However, variation experienced while *in utero* could have epigenetic consequences that may predispose some animals to having an impaired innate immune response leading to reduced health and less profitability for the producer, and limiting the accuracy of genetic selection for mastitis resistance.

Our hypothesis is that a large degree of between-animal variation in the innate immune response of dermal fibroblasts obtained from groups of calves or cows (Green, Kandasamy et al. 2011, Kandasamy, Green et al. 2011) is due to epigenetic variation. We aimed to investigate this through *in vitro* manipulation of cellular DNA methylation and histone acetylation. Modulation of epigenetic markers was accomplished using the chemical inhibitors 5-aza-2'deoxyctidine (AZA) and trichostatin A (TSA) that inhibit DNA methyltransferase (DNMT) and histone deacetyltransferase (HDAC) respectively. This treatment effectively reprograms the epigenetic makeup of the fibroblasts and removes animal-to-animal variation in epigenetic status. We subsequently compared the ability of cells treated with or without AZA, and with or without TSA, to recognize and respond to LPS. As well, we used genomic expression arrays to identify immune-responsive genes affected by the epigenetic modification. Our results indicate that DNA

methylation and histone acetylation are major causes of individual variation observed in the innate immune response of bovine dermal fibroblasts.

3.3 Materials and Methods

3.3.1 Dermal Fibroblast Cultures

All experiments were performed with approval of the Institutional Animal Care and Use Committee at the University of Vermont. Primary dermal fibroblast cultures were selected from a collection of cultures obtained from 15 sixteen-month old Holstein heifers for which the fibroblast collection, isolation, and ability to produce IL-8 in response to LPS has previously been described (Green, Kandasamy et al. 2011). Fibroblast cultures were selected based on production of IL-8 in response to LPS exposure, with the three lowest responding (LR) and three highest (HR) responding cultures chosen for further investigation.

3.3.2 Fibroblast Epigenetic Treatment and LPS Challenge

Following recovery from cryopreservation and expansion in a T-75cm² flask, cells were trypsinized and seeded in 6-well culture plates at 3.0×10^4 cells/mL in a total volume of 2 mL. Cells undergoing epigenetic treatment were either exposed to 5-aza-2'deoxyctidine (AZA; Sigma), trichostatin A (TSA; Sigma) or a combination of the two to achieve DNA demethylation, histone hyperacetylation, or both, respectively. Cells undergoing DNA demethylation were cultured for 96 hours in the presence of 10 μ M AZA. Cells selected to undergo histone hyperacetylation were cultured for 72 hours in plain medium at which point 80 nM TSA was added for 24 hours. Finally, for

cells undergoing both treatments, 10 μ M AZA was added again for 96 hours. At the 72-hour time point, 80 nM TSA was included for the final 24 hours. Control cells were grown for 4 days with comparable amounts of the AZA-TSA diluents (PBS and DMSO, respectively) added. Dosages and exposure time to AZA and TSA were modeled after previous experiments conducted that displayed low cytotoxicity from treatment in conjunction with effective epigenetic remodeling at similar or lower concentrations than those used in our trials (Takahashi, Sugi et al. 2009, Tsai, Li et al. 2012, Duijkers, Menezes et al. 2013)

Following epigenetic modification, cells were washed 3X with PBS and exposed to LPS for 24 hours. LPS treatment consisted of growth media supplemented with 100 ng/mL of ultra-pure LPS isolated from *Escherichia coli* O111:B4 (Sigma-Aldrich). Following appropriate exposure time, media was removed, spun at 500 x g for 5 minutes to remove cell debris, and immediately stored at -20°C until further analysis.

3.3.3 Quantification of IL-8

The concentration of IL-8 in fibroblast conditioned media was quantified by a custom sandwich ELISA previously described (Kandasamy, Green et al. 2011) using mouse anti-bovine IL-8 monoclonal antibody (clone 170.13 generously gifted by S. Maheswaren, University of Minnesota) and biotinylated goat anti-human IL-8 antibody (R&D Systems Inc., Minneapolis, MN) as capture and detection antibodies, respectively, and recombinant bovine IL-8 (Thermo Scientific, Rockford, IL) as assay standard. The detection limit of the assay was approximately 300 pg/ml.

3.3.4 Gene Expression Analysis Following AZA-TSA Treatment

The three fibroblast cultures with the lowest response to LPS treatment were selected for genome-wide expression analysis following AZA-TSA treatment using the Affymetrix custom bovine GeneChip array version1.0 (Affymetrix, Santa Clara, CA). This platform consists of 24,342 gene-level probe sets without replicates. Poly-A controls used were *dap*, *lys*, *phe*, and *thr* while hybridization controls were *bioB*, *bioC*, *bioD*, and *creX*. Following recovery from cryopreservation, cells were expanded and seeded into 6-well plates and kept under control conditions or treated with AZA and TSA as previously described. Fibroblasts were then exposed to LPS (100 ng/mL) for 0, 2, 4, or 8 hours, after which cell lysates were prepared for RNA isolation using a PurePerfect Kit (5 Prime, Gaithersburg, MD). On-column DNase digestion was performed to remove DNA during the RNA isolation procedure. Quality and quantity of the isolated RNA samples was determined with a 2100 Bioanalyzer (Agilent). Subsequently, gene expression analysis was conducted at the University of Vermont's core microarray facility. A total of 24 microarray slides were probed using RNA from three different LR animals. For each animal, sets of four paired cultures were either pre-exposed to AZA-TSA (as above) or held under control conditions for an equivalent length of time. After exposure to LPS (100 ng/ml) for 0, 2, 4, or 8 hours, RNA was collected for transcriptome analysis (GEO accession number GSE50405). Hybridization mixes were prepared and hybridized to the arrays as per manufacturer's recommendations using the Affymetrix 450 fluidics station and scanned with the Affymetrix 3000-G7 scanner.

3.3.5 Microarray Statistical Analysis

Probe statistics (CEL files) were calculated from scanned images using Affymetrix GCOS software. All other calculations were performed using Partek Genomics Suite tools (Partek, Inc., St. Louis, MO). Probe set and sample matrix expression statistics were calculated using the Robust Multichip Average (RMA) method (Irizarry, Hobbs et al. 2003). Sample quality was assessed based on the 3':5' ratio, relative log expression (RLE), and normalized unscaled standard error (NUSE). Principal Component Analysis (PCA) was also used to look for outlier samples that would potentially introduce latent variation into the analysis of differential expression across sample groups, of which none were discovered. Linear modeling of sample groups was performed using ANOVA as implemented in Partek Genomic Suites. The magnitude of the response (fold change calculated using the least square mean) and the p-value associated with each probe set and binary comparison were calculated, as well as a step-up, adjusted p-value for the purpose of controlling the false discovery rate (FDR).

When analyzing the effects of AZA-TSA on the cultures, genes were considered differentially expressed in comparison to controls if they passed the $FDR < 0.01$ and fold-change ≥ 1.5 thresholds. To determine the effects of LPS on gene expression, cultures at 2, 4, and 8 hour time points were compared to 0h cultures. Genes were considered differentially expressed if they passed the $FDR < 0.05$ and fold-change ≥ 1.5 thresholds. The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used for functional annotation and analysis by uploading Affymetrix-

based nomenclature of statistically significant genes (1.5-fold expression cut-off; FDR<0.05; <http://david.abcc.ncifcrf.gov>).

3.3.6 Quantitative Real-Time PCR

A subset of genes identified by microarray analysis was selected for expression analysis by quantitative real-time PCR (RT-PCR) using oligonucleotide primers specific to *TLR4*, *IL-8*, *IL-6* (Pareek, Wellnitz et al. 2005), *serum amyloid-A (SAA)-3*, *TNF- α* , and *CC-motif ligand (CCL) 20* (**Table 1**). The same RNA samples used in the genome-wide expression study were used. First-strand cDNA synthesis was conducted using the Improm II Reverse Transcriptase Kit (Promega). Messenger RNA expression was quantified by RT-PCR with a CFX96 Real-Time Instrument (Bio-Rad, Hercules, CA) using PerfeCTa SYBR Green Super-Mix, Low ROX kit (Quanta Biosciences). Cycling conditions were: initial denaturation at 95° C for 2 minutes; then 40 cycles consisting of denaturation at 95° C for 15 seconds, annealing at 60° C for 30 seconds and extension at 72° C for 1 minute. Melt curve analysis was also performed to check amplification of the desired gene product. The *β -actin* gene was used as reference gene for normalization procedure. Threshold cycles (Ct) generated using RT-PCR experiment for each sample were analyzed using the delta Ct method.

3.3.7 Statistical Analysis

IL-8 protein production was analyzed between groups (LR vs. HR) using a Student's t-test (Graph Pad Prism 5.0). Analysis of real time gene expression data was conducted using a two-way ANOVA model with repeated measures (Graph Pad Prism

5.0). Comparisons with $P < 0.05$ were considered statistically significant within experiments.

3.4 Results

3.4.1 Response to LPS Following Epigenetic Modification

IL-8 was undetectable prior to the addition of LPS in both the control and AZA-TSA cultures (data not shown). The fibroblasts subsequently produced substantial quantities of IL-8 in response to LPS treatment (**Figure 1**). In cells cultured under standard growth conditions and then exposed to LPS for 24 h, approximately twice as much ($P < 0.05$) IL-8 was produced by HR cells in comparison to LR cells. Removal of DNA methylation by AZA and hyper-acetylation of histone proteins by TSA alone or in conjunction led to an increased responsiveness to LPS evident by substantial increase in the production of IL-8 following LPS treatment (**Figure 1**). The AZA and/or TSA pre-treatments potentiated the LR culture responses more than the responses of HR cultures such that an overall loss of difference between groups was observed across all epigenetic treatments. There were no differences in the number of cells present for the LPS challenge between control and epigenetic modification cultures indicating that the treatment did not affect cell growth or division rates (data not shown).

3.4.2 Loss of Gene Expression Differences Between LR and HR Groups Following Epigenetic Modification

Real-time PCR was used to compare gene expression of three LR and three HR cultures following exposure to LPS (100 ng/mL) under standard and AZA-TSA treatment conditions. LPS up regulated *IL-8* in both LR and HR groups under standard

conditions, however at the 3-hour time point the HR group had higher ($P < 0.05$) expression than LR group (**Figure 2A**). In comparison, cells having undergone epigenetic modification had enhanced *IL-8* expression at the time of LPS addition, with further induction following LPS, but showed no between-group differences ($P = 0.46$) following exposure to LPS (**Figure 2B**). Similarly, an induction of *IL-6* following LPS was observed in all groups, with HR expression of *IL-6* being higher ($P < 0.01$) than LR cultures at 3 hours under control conditions (**Figure 2C**), and yet, following epigenetic modification there were no between-group differences in *IL-6* message at any time point (**Figure 2D**). Finally, *TNF- α* expression was induced by LPS, but was not significantly different between groups at any time point following LPS exposure under control or treatment conditions (**Figures 2E and 2F**).

3.4.3 Transcriptomic Analysis of Epigenetic Modification

Global gene expression analysis was compared between three LR cultures under standard or epigenetic modification conditions, at 0, 2, 4, and 8 hours following LPS exposure. Only LR cultures were selected due to their greater potentiation of the LPS-induced *IL-8* production response following epigenetic treatment as shown in Figure 1. The most sensitive cultures would potentially be more suitable to reveal genes affected by epigenetic modification.

In the absence of AZA-TSA, the cultures responded to LPS with a considerable number of genes altered at each time point following exposure. Within 2 hours of LPS exposure, 241 genes had altered expression (fold change ≥ 1.5 ; FDR < 0.05) in comparison to hour 0 levels. Gene expression measured 4 hours post-LPS indicated 278

genes (**Supplemental File 1**) displaying differential expression in comparison to hour 0; this number dropped to 137 genes by hour 8. Immune associated genes showing a strong response to LPS included *CCL5*, *chemokine CXCL motif (CXCL)-2*, *IL-6*, *IL-8*, and *TNF- α* among others (**Table 2**).

Analysis of the transcriptome was characterized by differential expression (fold change ≥ 1.5 ; FDR < 0.01) of 1406 transcripts (**Supplemental File 2**) due to epigenetic modification prior to LPS treatment (hour 0). Of genes associated with the innate immune response, several were up regulated by more than five-fold due to treatment alone (hour 0) including *CCL-17*, *CXCL2*, *IL-8*, and other genes including *ubiquitin-like modifier ISG15 (ISG15)*, *myxovirus (MX) resistance-1*, and *2'-5'-oligoadenylate synthetase (OAS)1* (**Table 2**). In addition, multiple genes involved in spermatogenesis as well as those located on the X chromosome were upregulated following AZA-TSA treatment.

Across all time points 1,758 transcripts were differentially expressed (fold change ≥ 1.5 ; FDR < 0.01) due to epigenetic modification, although, some of these genes showed little difference at hour 0 (262 genes FC < 1.5). This suggests a large regulatory role for methylation and acetylation in innate immune signaling. Within the group of genes showing differential expression between control and AZA-TSA treatments following LPS exposure, several genes associated with TLR4 signaling were up regulated including *IL-6*, *TNF- α* , *CXCL2*, *CCL20*, and *SAA3*, among others. These genes, excluding *CXCL2*, showed no differences in expression between cells grown under standard or AZA-TSA treatment conditions at hour 0. Many genes, such as *TLR4*

and *CXCL12* showed no differences in expression due to epigenetic modification.

Functional analysis of genes differentially expressed between control and AZA-TSA cultures at hours 2, 4, and 8 post-LPS (**Supplemental File 3**) using DAVID identified 12 pathways affected by treatment. These included p53 pathway signaling, fatty acid metabolism, focal adhesion, cytoskeleton regulation, and the cell cycle, among others.

3.4.4 Validation of microarray results by RT-PCR

Use of real-time PCR confirmed that epigenetic modification of LR cultures with AZA and TSA increased gene expression of *IL-8*, *IL-6*, *TNF- α* , *CCL20*, and *SAA3* ($P < 0.05$) at varying times following exposure to LPS (**Figure 3**). RT-PCR as a more sensitive probe of gene expression showed higher differential expression levels based upon treatment than did microarray analysis. Higher *IL-6* expression due to treatment as determined by microarray increased at hours 0, 2, 4, and 8 from 1.5, 2.5, 2.4, and 1.9 fold respectively to 3.1, 4.8, 4.2, and 4.7 fold. Fold change of *TNF- α* expression due to treatment as determined by microarray was 1.2, 1.7, -1.04, and -1.1 as compared to 3.2, 13.5, 3.8, and 15.0 fold higher by RT-PCR. A similar increase in differential expression of *CCL20* due to treatment was seen comparing results of microarray analysis (1.2, 3.8, 4.4, and 4.4 fold higher) and RT-PCR analysis (4.0, 16.7, 10.3, and 28.4 fold higher). Differential expression of *IL-8* increased dramatically when analyzed by RT-PCR, especially at the 0 hour time point. While microarray analysis revealed that *IL-8* expression was 6.6, 5.0, 7.3, and 4.3 fold higher at hours 0, 2, 4, and 8 respectively, RT-PCR analysis found *IL-8* expression to be 831, 14.0, 25.5, and 13.5 fold higher due to AZA-TSA treatment.

RT-PCR also confirmed that *TLR4* expression was not different ($P = 0.52$) between treatment and control cultures (**Figure 3E**). Analysis also showed that the fibroblast cultures did not increase expression ($P = 0.68$) of *TLR4* following exposure to LPS.

3.5 Discussion

The ability of an individual to recognize and respond to bacterial components such as LPS during an infection plays a critical role in host defense (Beutler, Hoebe et al. 2003). Previous evidence has indicated that variation in innate immune response capability such as a defects in the TLR4 signaling pathway may lead to higher disease susceptibility and poorer outcomes (Medvedev, Lentschat et al. 2003, Ku, von Bernuth et al. 2007). While our understanding of genetic variation and its role in the innate immune response has been informative, the interface between genetics and environment has been shown to play an important role in this pathway as well (Knight 2013). A better understanding of how individual variation is regulated across a population could lend to further discoveries of markers playing important roles in disease resistance.

With the advance of sequencing technologies, a large degree of epigenomic variation at the population level has been described across many organisms (Milosavljevic 2011). The goal of this study was to investigate the role of epigenetic variation on the innate immune response of bovine dermal fibroblast cultures from two phenotypically diverse populations. Establishing a narrowed epigenetic basis for variation in the response to LPS would focus investigations aimed at identifying

important biomarkers of the innate immune system. Our study focused on TLR4 signaling by using LPS responsiveness as the investigated phenotype, however it is likely that phenomenon observed here through epigenetic manipulation are more broadly applicable considering common downstream mediators shared between various PAMP and ligand receptors including the family of TLR receptors, IL-1R, and TNF-R (Verstrepen, Bekaert et al. 2008).

The use of epigenetic modifiers has previously been reported to alter cellular response to LPS through a shift in IL-8, TNF- α , and IL-6 production (Chavey, Mühlbauer et al. 2008, O'Gorman, Colleran et al. 2010, Gillespie, Savic et al. 2012). IL-8 is an important chemo-attractant of neutrophils and may be produced by a wide array of cell types following an infection (Andersen 2007, Lahouassa, Moussay et al. 2007, Herndon, Foreyt et al. 2010). IL-6 and TNF- α play roles in inflammation and the strength of production following infection has been linked closely to pathogen clearance (Zanotti, Kumar et al. 2002). The importance of these and other cytokines in the response to infections highlight the importance of understanding how variability in their production contributes to disease progression. The present study focuses on how alteration of epigenetic markers may affect between-animal variation in the immune response. The presence of AZA and TSA dramatically increased cellular responsiveness to LPS, without altering TLR4 expression, indicating an inhibitory role of DNA methylation and acetylation acting on the TLR4 signaling pathway. In addition, this increase in LPS responsiveness was greater in LR than HR fibroblasts. Under AZA, TSA, or AZA-TSA conditions, the differential response to LPS between

LR and HR cultures observed under standard conditions was abolished suggesting between-group differences in DNA methylation and acetylation as causative for the different IL-8 response phenotypes observed under standard conditions. The differential change between the extreme phenotypes appears to indicate that a high degree of variation exists in epigenetic modulators of gene expression across populations. Previous work has focused on epigenetic variation comparing healthy individuals to those affected by a specific disease (Andia, de Oliveira et al. 2010, Ishida, Kobayashi et al. 2011, Gillespie, Savic et al. 2012); our data aims to identify variability in the general population of cows that could affect susceptibility to a large number of pathogens. While IL-8 protein and mRNA variation between the pre-selected groups was expected, we also identified differential expression of *IL-6* between LR and HR cultures following LPS exposure for 3 hours. *TNF- α* expression was not different ($P = 0.08$) between groups likely due to greater variability between individuals within each group. We and others have shown that the timing of *TNF- α* induction following LPS is much more acute and short-lived than other LPS-responsive genes (Lee, Bannerman et al. 2006, Carroll, Reuter et al. 2009). Following treatment with AZA and TSA, the between-group differential expression of *IL-8* and *IL-6* was lost. The overall effect of AZA and TSA on the response of both LR and HR fibroblasts to LPS suggests that cultures from either group are heavily regulated by both methylation and acetylation. Both LR and HR cultures, in response to epigenetic modification, displayed significantly higher responses to LPS indicating that it is likely differing degrees of

epigenetic regulation between groups rather than a total absence in HR cultures that leads to the different phenotypes seen.

Microarray analysis of gene expression comparing control and AZA-TSA cultures identified over 1,700 genes that were differentially expressed due to the epigenetic modifications. It is important to note however, many of these genes (845 genes) were upregulated (Fold Change ≥ 1.5 ; FDR < 0.01) or repressed (561 genes) by AZA-TSA alone prior to LPS exposure. For the upregulated genes this likely reflects the loss of transcriptional repression due to DNA methylation and histone acetylation. When accounting for the interaction of AZA-TSA with LPS exposure, 828 genes were significantly (FDR < 0.01) altered. This large subset of LPS responsive genes modified by AZA and TSA suggests that epigenetic markers heavily regulate a large number of them. Genes responsive to our treatments typically fell into two general profiles. The first included genes that were up regulated by AZA-TSA before the addition of LPS into culture. In this group, expression increased without any other external stimulus being required indicating that under control conditions their expression was repressed by epigenetic markers. Immune associated genes such as *IL-8*, *CXCL2*, and *MX1* fell into this category. This correlates with previous data showing a similar increase in *IL-8* expression levels due to methylation and acetylation modifications alone, prior to any other stimulus (Angrisano, Pero et al. 2010). IL-8 protein was undetectable in AZA-TSA cultures following 24-hour incubation in control media (lacking LPS) suggesting that while transcript levels had risen, protein levels were still below the ELISA limit of detection. This may be due to an increase in transcript levels due to AZA-TSA

exposure without the requisite transcript stabilization associated with a response to LPS controlled by the p38 mitogen-activated protein kinase (MAPK) pathway (Hoffmann, Dittrich-Breiholz et al. 2002, Li, Kartha et al. 2002). This would account for the high transcript levels seen prior to the addition of LPS, but a lack of protein due to an unstable transcript before to translation. A second general response includes genes upregulated due to epigenetic modification, but only following exposure to LPS. These genes were seemingly unaffected by AZA-TSA treatment alone, and required a secondary stimulus (LPS) for differences to be seen. This profile would seem to indicate that our treatment removed transcription inhibitors, but did not lead to gene induction. A striking example of this is seen with *SAA3*, which was largely unresponsive following LPS exposure in standard conditions but was markedly up regulated by LPS following AZA-TSA treatment. This would suggest that without AZA-TSA, up-regulation of *SAA3* transcription is delayed due to requisite removal of epigenetic regulators blocking signaling. The typical *SAA3* response profile following innate immune stimulation is delayed by several hours compared to more acute responses of inflammatory cytokines (Larson, Weber et al. 2005).

Although an appreciable number of genes were affected by the epigenetic treatment in the response to LPS, it is interesting to note that many of them are downstream response proteins such as cytokines and chemokines while genes within the TLR4 signaling pathway were largely unaltered. Previous work investigating differential gene expression of high and low responsive dermal fibroblast cultures showed no differences in TLR4 signaling, but significant variation between groups in

downstream response genes (Kandasamy and Kerr 2012). In addition to genes involved in the innate immune response, functional analysis showed genes altered by AZA-TSA exposure following LPS exposure were associated with a number of pathways including cell adhesion, cell cycle, and fatty acid metabolism. This would suggest that AZA and TSA are having broad effects on our culture system to a greater extent than simply the immune response and TLR4 signaling.

General mechanisms to account for differential responsiveness between LR and HR groups include one of two potential mechanisms. One would be simply through a differential regulation in the transcription of LPS responsive cytokines such as *IL-6*, *IL-8* and *TNF- α* . Another may involve an indirect mechanism such as differential kinase activity, which is key to phosphorylation and activation of the mediators found in the response prior to NF- κ B activation such as MyD88 or interleukin-1 receptor associated kinase (IRAK-1). This would not require differential expression of these mediators, but would still lead to differences in activation of NF- κ B responsive genes.

Among the genes affected by AZA-TSA treatment before the addition of LPS, a number control for X chromosome inactivation and spermatogenesis. It is interesting to note as all samples were from females, and may be used to highlight the importance of epigenetic silencing in sex differentiation. In females, a single X chromosome is silenced to ensure that X gene expression is not doubled in comparison to males (Kelkar and Deobagkar 2010). Several X antigen family genes, including member 5, were up regulated as well as *chromosome X open reading frames 23, 26, and 57*. The gene *X-inactive specific transcript (Xist)* has been implicated as a master control for X

chromosome inactivation (Jeon, Sarma et al. 2012), and while upregulated due to AZA-TSA in our model, it appears as though DNMT and HDAC inhibition played a larger role in the reactivation of the silenced X genes. Prior research has hypothesized that errors in the epigenetic silencing of the X chromosome may cause autoimmune diseases and rheumatoid arthritis (Chabchoub, Uz et al. 2009). While this may be the case, it is difficult to form a strong connection between the increase in X inactivated genes and the heightened response to LPS in our model. In addition to X inactive genes, several involved in spermatogenesis including *deleted in azoospermia-like (DAZL)*, *spermatogenesis associated 22 (SPATA22)*, *spermatogenesis and oogenesis specific basic helix-loop-helix 2 (SOHLH2)*, and *sperm acrosome associated 1 (SPACA1)* were highly upregulated due to AZA-TSA treatment. Again, while important to note as sex determinant genes heavily regulated by epigenetic silencing, it is unlikely that they played a major role in the alteration of the innate immune response within our data set.

While important markers of the immune response were investigated in relation to epigenetics, this study did not attempt to define specific areas of variation leading to the phenotypic differences described. AZA and TSA are global modulators of epigenetic markers and while effective in reducing variation, their use makes it difficult to pinpoint a specific area important in this effect. Future studies should focus on where differences in DNA methylation or histone acetylation occur between low and high responsive cultures to better define potential mediators of this variable response to LPS.

3.6 Conclusion

This study is an important first step towards the understanding of epigenetics and individual variation in the innate immune response via TLR4 signaling. The loss of variation between low and high LPS responsive cultures in IL-8 production and *IL-6* transcription due to epigenetic reprogramming suggests that a majority of phenotypic variation is regulated by this mechanism. In addition the description of genes within our model modified by AZA and TSA treatment in the response to LPS helps to define areas of the innate immune response regulated by epigenetic markers. Increasing our understanding of the epigenetic regulation of LPS response will make the identification of potential environmental stimuli affecting disease susceptibility more realistic. In doing so, this knowledge may be an important key to reducing disease susceptibility in both production animals as well as humans.

Supplemental Files

Supplemental files from this chapter are publicly available and may be found at:

www.sciencedirect.com/science/article/pii/S0165242713002924

Table 1. Primer pairs used for amplification of target genes by RT-PCR.

Gene	Forward Primer Sequence	Reverse Primer Sequence
IL-8	GCTGGCTGTTGCTCTCTTG	AGGTGTGGAATGTGTTTTTATG
IL-6	TGAGGGAAATCAGGAAAATGT	CAGTGTTTGTGGCTGGAGTG
TNF	TCTTCTCAAGCCTCAAGTAACAAGC	CCATGAGGGCATTGGCATAAC
SAA3	CCTCAAGGAAGCTGGTCAAG	TACCTGGTCCCTGGTCATAAC
CCL20	TTCGACTGCTGTCTCCGATA	GCACAACCTGTTTCACCCACT
TLR4	ACTGCAGCTTCAACCGTATC	TAAAGGCTCTGCACACATCA
β -Actin	GCAAATGCTTCTAGGCGGACT	CAATCTCATCTCGTTTTCTGCG

Table 2. Expression of selected genes either responsive to LPS or exhibiting differential expression due to AZA-TSA treatment.

Gene Symbol	AZA-TSA vs Control (Hour 0)	Control Conditions (vs. Hour 0)			AZA-TSA (vs. Hour 0)		
		Hour 2	Hour 4	Hour 8	Hour 2	Hour 4	Hour 8
Chemokines							
CCL17	8.3**	1.0	1.0	-1.1	1.0 [@]	1.1 [@]	1.1 [@]
CCL2	3.3**	27.5**	20.7**	19.0**	16.8**	13.8**	9.9**
CCL20	1.2	1.4	1.5	1.3	4.4** [@]	5.6** [@]	4.6** [@]
CCL5	1.2	1.5	1.8*	1.9*	2.6** [@]	2.4**	2.5**
CXCL1	10.4**	100.1**	44.7**	40.9**	11.8**	8.4**	8.2**
CXCL2	25.8**	89.9**	69.9**	58.4**	6.2** [@]	6.7** [@]	5.9** [@]
CXCL3	4.3*	40.9**	12.2**	10.3**	15.7**	7.5** [@]	5.9**
CXCL5	4.1*	4.5**	8.9**	10.6**	2.2**	3.6** [@]	4.8**
Cytokines							
IL1A	1.3	4.3**	2.6	1.9	7.2**	3.9**	2.3
IL1F6	1.2	1.4	2.1	3.4**	1.5	2.8*	2.5*
IL-1RN	3.2**	1.3	1.2	-1.1	1.3 [@]	1.4 [@]	-1.1 [@]
IL6	1.5	6.8**	4.9**	5.1**	11.6** [@]	8.2** [@]	6.7**
IL8	6.6**	10.2**	4.7**	9.2**	7.8** [@]	5.3** [@]	6.0** [@]

TNF	-1.3	1.9*	1.6	1.3	2.7**	1.3	-1.1
Anti-viral							
IFIH1	8.1**	1.0	2.4*	3.3**	1.36 [@]	1.38 [@]	1.7* [@]
ISG15	24.8**	1.9	2.4*	3.6**	1.14 [@]	1.20 [@]	1.3 [@]
OAS1	47.8**	1.9	2.7*	7.6**	1.29 [@]	1.26 [@]	1.7 [@]
MX1	25.3**	1.1	1.5	2.8*	1.12 [@]	1.15 [@]	1.3 [@]
TLR Response Pathway							
CD40	1.2	2.2*	2.6*	2.5*	1.9	2.0*	2.0*
NFKB2	-1.2	1.5*	1.5*	1.5*	2.2**	2.3**	2.0**
NFKBIA	-1.0	7.7**	3.9**	3.3**	9.5**	5.9**	3.4**
NFKBIZ	1.1	5.8**	2.3*	2.2*	10.4**	4.7**	3.0**
Acute Phase Response							
SAA3	-1.1	1.2	2.1	3.1*	13.8* [@]	24.0* [@]	22.4* [@]
Down- regulated by AZA- TSA							
BRCA1	-2.0*	-1.3	-1.2	1.1	-1.2	-1.6	1.1
IRAK4	-1.4*	-1.3	-1.1	1.1	-1.1	1.0	1.2*
PTX3	-1.1	1.7*	2.3**	2.3**	1.7*	2.1**	1.9*

Data obtained by microarray analysis and represented as fold change comparing expression between control and AZA-TSA cultures at hour 0 or as fold change comparing hours 2, 4, and 8 within each treatment to expression at hour 0. A positive fold change indicates either higher expression in AZA-TSA cultures (0 hour comparison) or a higher expression than hour 0 (LPS response) **FDR < 0.01; * FDR<0.1; @ indicates differential expression between AZA-TSA and control culture at specified time point FDR<0.01.

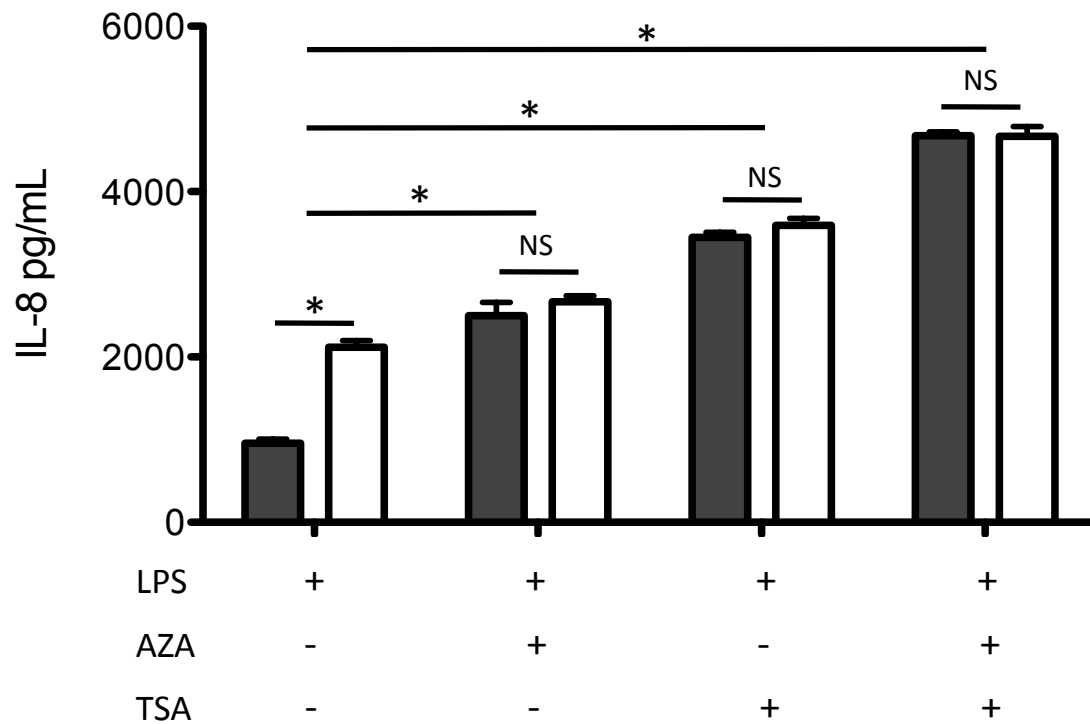


Figure 1. Fibroblast response to LPS under control conditions or following epigenetic modification.

Production of IL-8 by dermal fibroblasts following exposure to LPS (100 ng/ml) for 24 hours after undergoing pre-treatment with media alone, AZA, TSA, or AZA-TSA. Animals displaying differential responsiveness to LPS (Low responders (gray bars) and High responders (white bars); n=3/group) were investigated. In the absence of AZA and TSA, low and high responders showed differential responsiveness to LPS. However, following exposure to AZA and TSA either alone or in conjunction, differences in responsiveness to LPS were lost between the groups. In addition, exposure to AZA and TSA alone or in conjunction significantly increased IL-8 production in response to LPS. IL-8 production in the absence of LPS was not detectable. * indicated $P < 0.05$. All values are mean \pm S.D. (n=3/group).

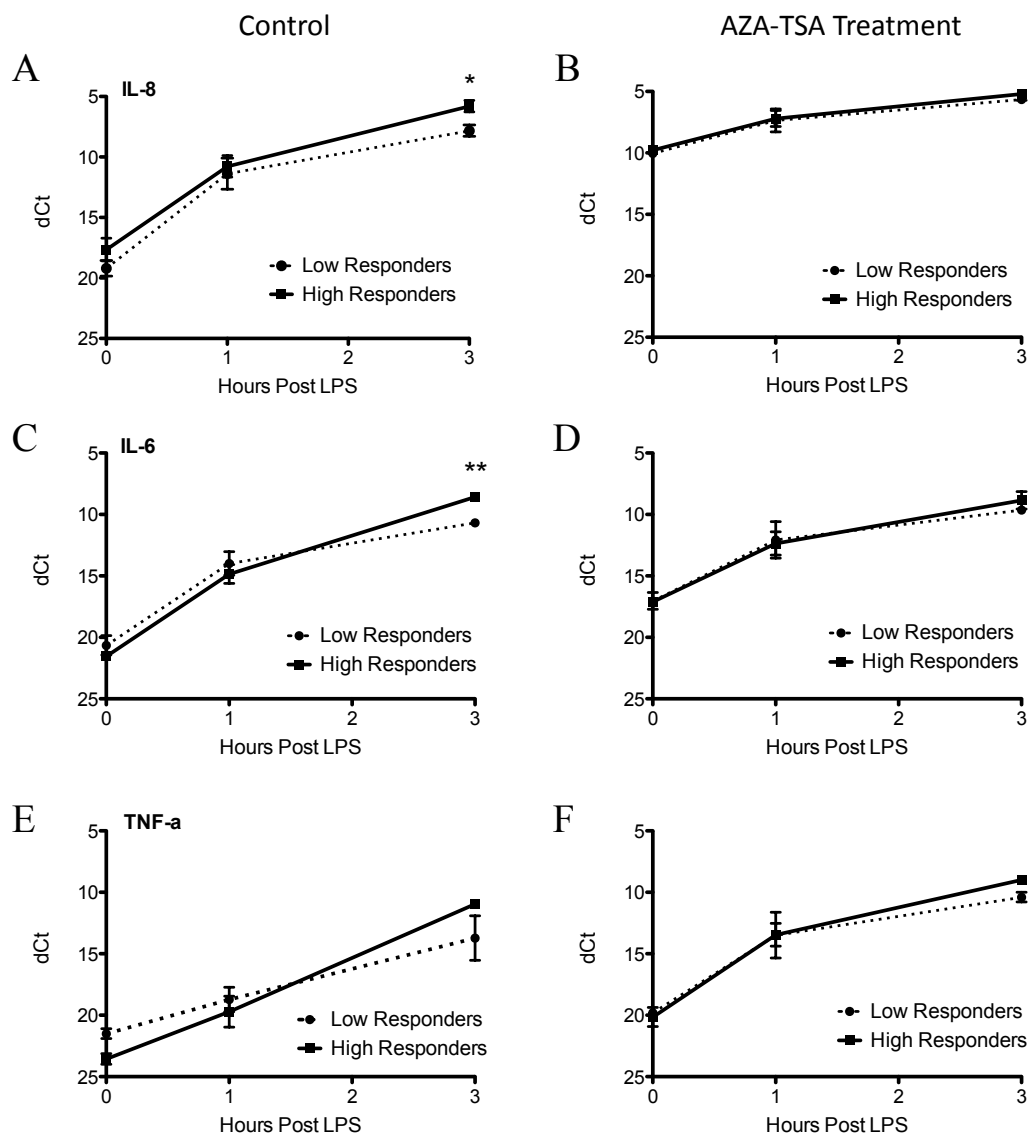


Figure 2. Fibroblast response to LPS as measured by RT-PCR under control conditions or epigenetic modification. Comparison of gene expression following exposure to LPS at hour 0 for low and high responding cultures under both control (left panel) and epigenetic modification (right panel) conditions. IL-8 (A and B), IL-6 (C and D), and TNF- α (E and F) mRNA were all targeted. Values are expressed as dCt using β -actin expression as the endogenous control. All values are mean \pm S.D. (n=3/group). * indicates significantly different expression levels ($P < 0.05$).

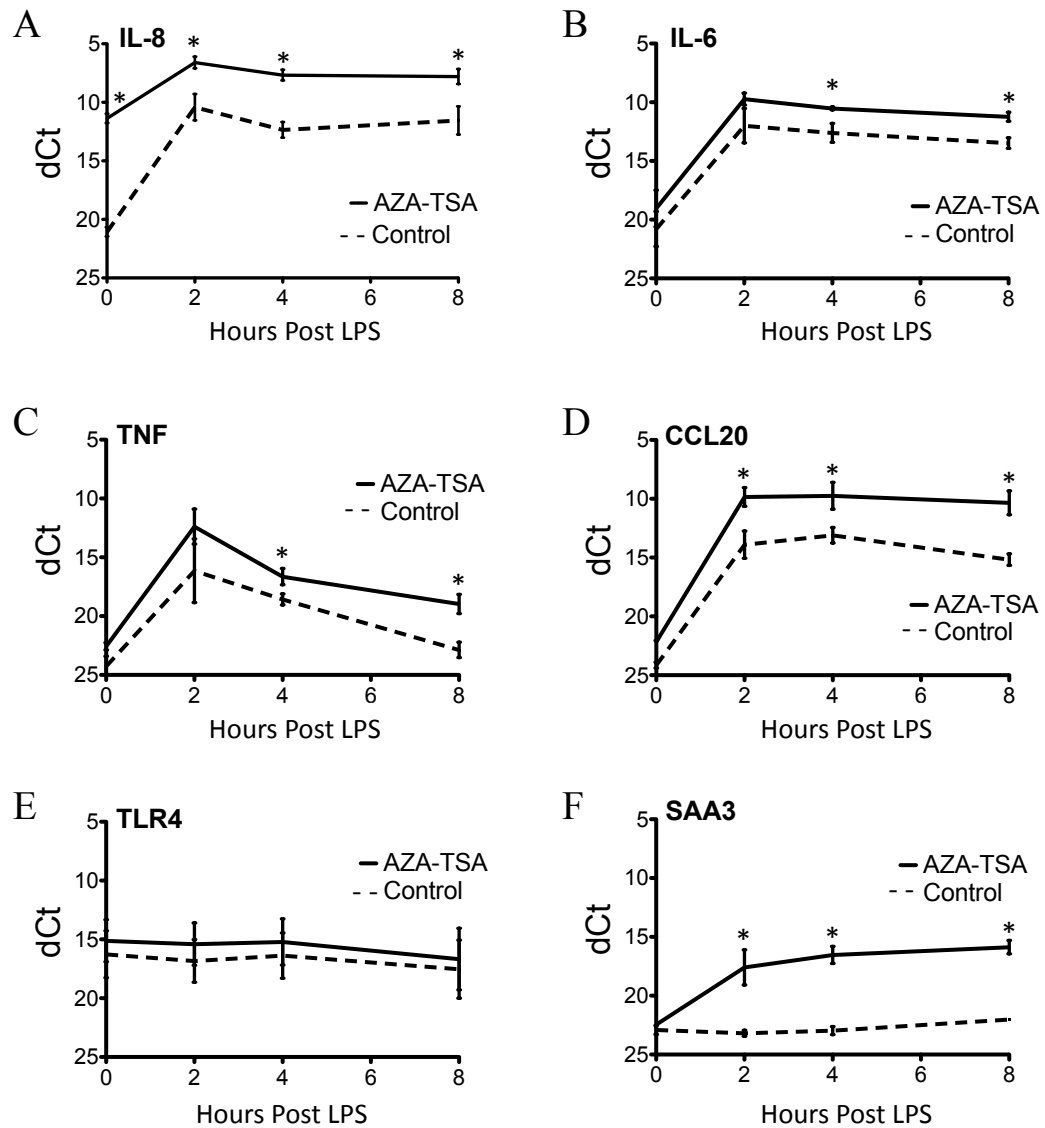


Figure 3. Confirmation of microarray by RT-PCR. Expression of IL-8 (A), IL-6 (B), TNF- α (C), CCL20 (D), TLR4 (E) and SAA3 (F) mRNA following exposure to LPS for three low responding cultures. Expression was analyzed for response under both control and epigenetic treatment conditions. * indicates significantly different ($P < 0.05$) gene expression due to epigenetic modification. Values are expressed as dCt using β -actin expression as the endogenous control. All values are mean \pm S.D.

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CHAPTER 4:

Age dependent changes in the LPS induced transcriptome of bovine dermal fibroblasts occurs without major changes in the methylome.

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4.1 Abstract

Background

By comparing fibroblasts collected from dairy cows at 5-months or 16-months of age we have previously found that the older cultures produce much more IL-8 in response to lipopolysaccharide (LPS) stimulation. We now expand this finding by examining whole transcriptome differences in the LPS response between young and older cultures, and also investigate the contribution of DNA methylation to the epigenetic basis for the age-dependent increases in responsiveness.

Results

Age-dependent differences in IL-8 production by fibroblasts in response to LPS exposure for 24 h were abolished by pretreatment of cultures with a DNA demethylation agent, 5-aza-2'deoxyctidine (AZA). RNA-Seq analysis of fibroblasts collected from the same individuals at either 5 or 16 months of age and exposed in parallel to LPS for 0, 2, and 8 h revealed a robust response to LPS that was much greater in the older cultures. Pro-inflammatory genes including IL-8, IL-6, TNF- α , and CCL20 (among many other immune associated genes), were more highly expressed (FDR<0.05) in the older cultures following LPS exposure. Methylated CpG island recovery assay sequencing (MIRA-Seq) revealed numerous methylation peaks spread across the genome, combined with an overall hypomethylation of gene promoter regions, and a remarkable similarity, except for 20 regions along the genome, between the fibroblasts collected at the two ages from the same animals.

Conclusions

The fibroblast pro-inflammatory response to LPS increases dramatically from 5 to 16 months of age within individual animals. A better understanding of the mechanisms underlying this process could illuminate the physiological processes by which the innate immune response develops and possibly individual variation in innate immune response arises. In addition, although relatively unchanged by age, we present a general overview of the bovine fibroblast methylome as a guide for future studies in cattle epigenetics.

4.2 Introduction

The innate immune response plays an important role in the clearance of pathogens following an infection, including during bovine mastitis. Components of the recognition and response pathway, mediated by toll-like receptor (TLR)4, are susceptible to regulation of gene expression by epigenetic mechanisms. For example, DNA-methylation of the *TLR4* gene promoter has been linked to a lowered response to LPS in intestinal epithelial cells (Takahashi, Sugi et al. 2009). Conversely, DNA hypomethylation has been implicated in over expression of *myeloid differentiation factor (MD)-2* in human intestinal epithelial cells leading to higher responsiveness to LPS exposure (Vamadevan, Fukata et al. 2010). Therefore, it may be postulated that phenotypic variation in the response to LPS between individuals may be partially controlled by epigenetic modification. Environmental exposures have been linked to

alteration in the innate immune response as well, with studies conducted on pregnant rats showing that prenatal exposure to LPS leads to a suppressed innate immune response in offspring when examined at 5 days post birth (Hodyl, Krivanek et al. 2008) or even after 40 weeks of life (Williams, Teeling et al. 2011). Understanding the role of environmental cues and subsequent epigenetic modifications in the variable immune response to LPS in the dairy cow would further our current understanding of disease susceptibility.

We have previously demonstrated an age-dependent increase in the immune response of bovine dermal fibroblasts (Green, Kandasamy et al. 2011), with cultures from collected the same individual at 16 versus 5 months of age showing an increase in IL-8 production in response to LPS. Understanding the potential epigenetic mechanisms regulating the development of the bovine innate immune response within an individual could be used to help understand underlying causes of variation between individuals. For example, dairy cows display a range of responses when exposed to the same bacterial pathogen in experimental mastitis challenge studies (Kornalijnslijper, Daemen et al. 2004, Buitenhuis, Rontved et al. 2011). We have also found a substantial range between animals in the magnitude of response of fibroblasts to LPS stimulation that relates to the in vivo response to intravenous LPS (Green, Kandasamy et al. 2011) or intramammary *E. coli* challenge (Kandasamy, Green et al. 2011). The use of fibroblasts collected from the same animal at different ages allows for the investigation of phenotypic variation without confounding genotypic differences.

One potential mechanism controlling the TLR response pathway may be DNA methylation. Some data exists on the role of DNA methylation affecting the TLR4 signaling pathway in humans (Takahashi, Sugi et al. 2009, Vamadevan, Fukata et al. 2010), though only limited data exists for dairy cows (Green and Kerr 2014). In addition, changes in DNA methylation with age have previously been described, further implicating it as a potential mechanism of age associated alterations in gene expression and innate immune response (Koch, Suschek et al. 2011, Martino, Tulic et al. 2011). Understanding DNA methylation's role in the alteration of the innate immune response would allow research to focus on environmental cues or therapeutic treatments that may skew a population towards a more desirable immune response phenotype, naturally increasing disease resistance.

While the bovine innate immune response has been well characterized under both *in vitro* and *in vivo* conditions (Ibeagha-Awemu, Lee et al. 2008, Rinaldi, Li et al. 2010, Green, Kandasamy et al. 2011), little research has been conducted to determine the development of the response to lipopolysaccharide due to age within an individual. In addition, though a factor with potentially broad implications in gene expression and disease, there is only a limited data set available detailing DNA methylation in the economically important bovine model. Much of the previous work investigating the development of the innate immune response was conducted using mixed cell models from different individuals, resulting in highly variable results (Liao, Yeh et al. 2013, Maniar-Hew, Clay et al. 2013). This study employed a pure cell culture model, and eliminated genotypic variation by isolating cells from the same individual at different

ages reducing much of the technical and biological variation that may have been present in previous studies offering a clear view of the age-dependent changes in TLR4 signaling.

This study aimed to map transcriptomic alterations in the TLR4 response pathway of bovine dermal fibroblasts isolated from the same individuals at different ages and to determine what effect changes in DNA methylation patterns have on this response. To accomplish this, RNA-Seq was performed on bovine dermal fibroblasts isolated from the same individuals at two different ages (5 months and 16 months) and exposed to LPS for 0, 2, and 8 hours. In addition, methylated CpG island recovery assay sequencing (MIRA-Seq) was performed on DNA from the same individuals collected at the two different ages. This project allowed for the first in depth analysis of transcriptome wide changes in gene expression due to age in the bovine fibroblast model while also creating the first descriptive study of the bovine fibroblast methylome and its potential role in altering the innate immune response within an individual over time.

4.3 Results

4.3.1 Fibroblast Challenge with LPS and Epigenetic Modification

Fibroblasts isolated from 15 dairy heifers at ~5 and 16 months of age have previously been described as showing an age-dependent increase in IL-8 production in response to LPS *in vitro* (Green, Kandasamy et al. 2011). To determine if DNA methylation may have contributed to this age effect, pairs of fibroblast cultures

collected at 5 and 16 month of age from three mid-level responding animals were selected for epigenetic modification using the demethylating agent AZA prior to LPS exposure for 24 h. Neither control nor AZA treated cultures produced detectable IL-8 in the absence of LPS. However, in response to LPS, young and old cultures displayed greater than 5-fold differences in IL-8 production ($P < 0.001$), with 5-month-old fibroblasts producing 240 ± 30 pg/ml in contrast to $1,350 \pm 290$ pg/ml produced by the older cultures (**Figure 1**). Following AZA treatment, the differential response between age groups was abolished ($P > 0.05$) with increased IL-8 levels of $1,580 \pm 50$ pg/ml and $1,690 \pm 70$ pg/ml produced by the young and old cultures, respectively. The young cultures in particular displayed much higher ($P < 0.01$) LPS-induced IL-8 production following AZA treatment.

4.3.2 RNA-Seq analysis of young and old response to LPS

Total RNA samples from each of the three animals, collected at two ages, and exposed to LPS for 0, 2, and 8 hours were prepared for RNA-Seq analysis. This analysis generated approximately 74 million reads per sample following quality control. Alignment to the UMD v3.1 bovine genome yielded 96% of reads falling within alignment parameters and thus an average of 71 million reads per sample. At hour 0, under our definition of expression (CPM > 1 in at least 50% of samples), 18,919 targets were detected forming the core transcriptome of the bovine dermal fibroblast under basal conditions. Following LPS exposure, 18,616 and 19,204 targets were detected after 2 and 8 hours, respectively. In comparison to hour 0, exposure to LPS revealed differential gene expression (FDR < 0.05 ; CPM > 1 ; Fold Change ≥ 2) of 617

transcripts at hour 2 (414 up- and 203 down regulated; **Additional File 1**) and 414 transcripts at hour 8 (260 up- and 154 down-regulated; **Additional File 2**). Among the genes displaying differential induction due to LPS exposure were those involved in the pro-inflammatory response including *IL-8*, *IL-6*, *CCL20*, *TNF*, and *CXCL2* among others (**Table 2**). In addition to pro-inflammatory cytokines, the presence of LPS induced many Type-I interferon related genes, including *Myxovirus (MX)-1*, *Ubiquitin-like gene (ISG)-15*, and *interferon induced with helicase C domain (IFIH)-1*, among others. This wide array of cytokines and interferon related genes demonstrate the ability of the dermal fibroblasts to recognize and respond to LPS.

Comparing expression between young and old cultures revealed 628, 280, and 920 differentially expressed genes (FDR < 0.05; CPM > 1; Fold Change \geq 2) at 0, 2, and 8 hours post-LPS exposure, respectively (**Figure 2; Additional File 3**). Of these differentially expressed genes, 400, 206, and 595 displayed higher levels in old cultures at hours 0, 2, and 8, respectively. The lower number of differentially expressed genes at hour 2 reflects greater inter-culture variation in response at this time point. Pathway enrichment analysis using DAVID indicated that at hour 2 genes within the inflammatory response ($P = 6.2 \times 10^{-9}$) and defense response ($P = 3.5 \times 10^{-8}$) pathways were the most strongly implicated as different between young and old cultures. Similar analysis at hour 8 implicated a larger subset of pathways as being differently expressed, however, inflammatory response ($P = 4.3 \times 10^{-6}$) and defense response ($P = 5.8 \times 10^{-7}$) were still different between the groups. Among the genes in these pathways displaying

differential expression between young and old cultures at either 2 or 4 hours post LPS were *IL-8*, *IL-6*, *TNF*, *CCL20*, and *SAA3* among others (**Table 3**).

In addition to genes related to the immune response, those that may be implicated in differential methylation levels were investigated. Among these are the family of *DNMT* genes along with *ubiquitin-like containing PHD and RING finger domains (UHRF)1*. Expression of *DNMT3A* and *3B*, which are involved in de novo methylation, showed no differences between young and old cultures. Expression levels of *DNMT1* and *UHRF1* however, involved in DNA methylation maintenance, were significantly higher in older cultures at all time points. At hour 0, *DNMT1* levels were 1.6 fold higher while *UHRF1* expression was 3.4 fold higher. These levels of differential expression were maintained at hours 2 and 8 post-LPS (**Table 3**).

4.3.3 Confirmation of RNA-Seq by RT-PCR

Several genes were selected for RT-PCR confirmation of expression differences between young and old cultures that were indicated by RNA-Seq analysis. *IL-8*, *TNF- α* , *TLR4*, and *CD14* showed consistent values in comparison to those from the RNA-Seq data set. The greatest difference in expression between young and old cultures for *IL-8* (**Figure 3A**) and *TNF- α* (**Figure 3B**) was at hour 2 post-LPS with 13.6 and 18.4 fold higher expression, respectively, in older cultures. By hour 8, older cultures were still producing higher levels of both *IL-8* (4.2 fold) and *TNF- α* (20.4 fold), but the differences between the young and old had been reduced. In contrast, *CD14* (**Figure 3C**) and *TLR4* (**Figure 3D**) showed little response to LPS, but displayed consistently higher expression in old cultures. For *TLR4*, old cultures displayed 4.8, 4.6, and 3.8 fold

higher expression at hours 0, 2, and 8. *CD14* followed a similar pattern, with 4.4, 6.7, and 4.6 fold higher expression in older cultures at 0, 2, and 8 hours, respectively.

CCL20 (**Figure 3E**) and *SAA3* (**Figure 3F**) displayed a strong response to LPS in both young and old cultures, but were more highly expressed in old cultures. No differences in expression were seen at hour 0, however older cultures produced 17.6 and 32.0 fold higher transcript levels of *CCL20* at hours 2 and 8, respectively. *SAA3* expression was not different between ages at hours 0 or 8, but at hour 2, older cultures produced 5.6 fold more transcripts. The gene expression values generated by RT-PCR were all in general agreement with RNA-Seq data, validating the transcriptomic results.

4.3.4 MIRA-Seq

The 6 MIRA-Seq samples from the young and old cultures resulted in an average of approximately 51 million reads per library following quality control. Alignment to the UMD v3.1 bovine genome yielded 96% of reads falling within alignment parameters and an average of 49 million mapped reads per sample. The genome was analyzed by construction of sequential 3kb regions in which the read count between young and old cultures were compared. Analysis of the 3kb genome scan yielded 20 regions that showed significantly different levels of methylation between groups ($FDR < 0.1$; $CPM > 1$; $FC \geq 2$). Of these DMRs, 13 displayed higher methylation levels in the young cultures, while 7 indicated higher methylation in older cultures. Of the 13 DMRs displaying higher methylation levels in younger cultures, 7 were found at least partially within an annotated gene while 3 of the 7 DMRs showing higher methylation in old cultures were within annotated genes (**Table 4**). No

differences were seen between young and old cultures however at the level of promoter methylation, as determined by our definition of -2500 bp to + 500bp from transcription start site. MIRA-Seq data was also used to construct a heat map of the bovine fibroblast methylome using the Circos software package to illustrate the genome-wide nature of DNA methylation (**Figure 4**).

4.3.5 The Role of DNA Methylation on Gene Expression

The association between methylation levels within a gene body or gene promoter region and that gene's levels of expression was analyzed by Fisher's exact test. MIRA-Seq values for a gene body or gene promoter were plotted against RNA-Seq expression levels at 8 h post-LPS. Each data point was binned as either being a high or low, with a gating value of RPKM=5 or RPKM=0.5 for RNA-Seq and MIRA-Seq, respectively. Analysis of the relationship between promoter methylation level and that gene's subsequent expression level showed that the two values were significantly ($P < 0.001$; O.R.=1.53; 95% C.I.=1.39-1.67) dependent upon one another, and indicated a strong inverse relationship between gene promoter DNA methylation and gene expression (**Figure 5A**). The relationship between gene body methylation levels and that gene's expression levels were also significantly ($P < 0.05$; O.R.=1.15; 95% C.I.=1.05-1.26) dependent upon one another, though to a smaller extent than promoter methylation (**Figure 5B**). The 8-hour time point was selected for presentation as this offered the period with the greatest number of genes showing high expression levels as analyzed by edgeR. Analysis of the 0 and 2-hour post-LPS times gave similar results (data not shown).

DNA methylation levels were also measured in relation to genomic region. Methylation was measured as RPKM in gene promoters, gene bodies, or intergenic regions and revealed that promoter regions have significantly less methylation than gene body or intergenic regions while these two showed no difference from one another (Figure 5C).

4.4 Discussion

The ability of an individual to recognize and respond to bacterial components such as LPS during an infection plays a critical role in host defense (Beutler, Hoebe et al. 2003). Previous work has suggested that neonates are deficient in innate immune responsiveness in comparison to adults (Kollmann, Crabtree et al. 2009, Nguyen, Leuridan et al. 2010) however these studies utilized mixed-cell models and were subject to highly variable responses. In using bovine fibroblast cultures, we are able to challenge a single population of cells isolated from the same animal at different times, but cryopreserved and exposed to LPS in side by side wells, eliminating much of the technical variability seen in these previous studies. A broader understanding of the regulated development of the innate immune response would potentially allow the application of this knowledge to determining factors that may be producing variability in the response of adult animals to different pathogens.

The goals of this study were to further our previous observation of age-dependent differential gene expression in response to LPS, and to determine what role DNA methylation may be playing in this process. By using dermal fibroblast cultures

collected from the same animal at different ages we were able to eliminate genetic variation as a confounding factor. We had previously found that fibroblast cultures from the same individual but collected at either five or sixteen months of age showed distinct differences in their ability to produce IL-8 in response to LPS, with the older cultures displaying a more hyper-responsive phenotype (Green, Kandasamy et al. 2011). In addition, we found that variability in the response between individuals could be abolished with the exposure of cultures to AZA, a global demethylating agent (Green and Kerr 2014). In the current experiment, we expand on these results to show that the age-dependent differential response to LPS as measured by IL-8 protein production by young and old individuals are also abolished by exposure of cultures to AZA. We also explored the bovine transcriptome to fully describe age-dependent differences in gene expression due to LPS exposure.

RNA-Seq data showed a great deal of differential expression between young and old cultures, with the greatest differences in LPS response seen in immune response pathways such as inflammation and cell defense. Many of the differentially expressed genes were cytokines and chemokines, previously shown to play an important role in the resolution of bacterial infections such as *E. coli* mastitis (Burvenich, Van Merris et al. 2003, Kandasamy, Green et al. 2011). In addition to *IL-8*, *IL-6*, *TNF- α* , and *CCL20*, many components of the LPS response pathway showed differential expression levels due to age. These included *TLR4*, the extracellular receptor of LPS, as well as *CD14*, an important co-receptor in the TLR4 signaling pathway (Jiang, Georgel et al. 2005). The differential expression of genes involved in

LPS recognition suggest that cultures from older animals are more readily able to detect danger signals by the binding of PAMPs. Following recognition of LPS, the interleukin-1 receptor associated kinase (IRAK) family of proteins, TIR domain-containing adaptor molecule (TRIF), and TNF receptor-associated factor (TRAF)-6, among others, act as downstream mediators of NF- κ B activation (Ostuni, Zanoni et al. 2010). Within our cultures, there appeared to be no up-regulation of these intermediates in the response to LPS, nor any increase in expression within an individual due to age. Many of these intermediates are activated by phosphorylation however, which may lessen the importance of changes to their expression levels from playing an integral role in NF- κ B activation. The TLR signaling pathway concludes with the activation of NF- κ B and activating protein (AP)-1 the subsequent up-regulation of immune response genes. Subunits of the NF- κ B complex, namely p50 and p52 showed higher expression (1.4 fold in both) in older cultures 8 hours post-LPS exposure, but were not differentially expressed due to age at 0 or 2 hours post-LPS. Since inflammatory markers such as *IL-6*, *IL-8*, and *TNF- α* were already differentially expressed between old and young cultures prior to the 8-hour time point, it does not appear that this could be a major factor in the differential response due to age.

Another cytokine of interest is IL-1 α , which can have a strong autocrine effect on cells producing it (Günther, Esch et al. 2011). In comparison to basal levels, fibroblast cultures showed strong induction of *IL-1 α* at 2 hours (86 fold induction) though by 8 hours post-LPS exposure the response had begun to decline (23.5 fold induction). Also of interest is the fact that older cultures expressed 2.3 and 2.8 fold

higher levels than young cultures at 2 and 8 hours post-LPS respectively. Intracellular IL-1 α has been linked to increased sensitivity to inflammatory stimuli by NF- κ B and AP-1 suggesting that it plays an important role in the modulation of the inflammatory response (Werman, Werman-Venkert et al. 2004). This increase in IL-1 α among the many other pro-inflammatory cytokines and chemokines in older cultures point towards a shift in the expression of immune response related genes due to age alone in many individuals. Related research has shown responsiveness to LPS exposure in airway epithelia of rhesus monkeys, as measured by IL-8 protein production as well as IL-1 α transcript, increased in an age dependent manner (Maniar-Hew, Clay et al. 2013). Research on the response to LPS in human monocytes and dendritic cells confirmed an increase in the TLR signaling response with age as responsiveness of infants increased over time reaching adult levels within one year of age (Nguyen, Leuridan et al. 2010). The gene expression data presented here, along with the work conducted by others suggests that individuals undergo a distinct maturation of response to LPS.

The overall response to LPS as measured here by RNA-Seq agreed with our previously published data on the response of selected low responding fibroblasts to LPS as measured by microarray (Green and Kerr 2014). Though that experiment focused on individuals with a low responding phenotype in comparison to this study using mid-level responding cultures, the overall profile of genes activated following endotoxin exposure (with the exception of SAA3) were similar. Up regulation of SAA3 is often a slow process (Larson, Weber et al. 2005) and the differences between microarray and RNA-Seq may be due to temporal differences in response between low and mid-

responding cultures rather than technical variation between the two gene expression analyses.

The RNA-Seq data also presents a comprehensive view of the temporal aspects of the LPS response. There are several hundred genes displaying age-dependent differential expression under basal conditions (hour 0) presenting a picture of the varying landscape of genetic regulation within an individual due to age in comparison to our previous microarray study, which focused only on low responding cultures. However, after 2 h of LPS exposure there is a marked reduction in the absolute number of differentially expressed genes due to high levels of variability across the cultures sampled. This would appear to indicate that within the first two hours of LPS exposure, the machinery controlling gene expression is in flux that limits discernable bias between young and old cultures in the speed in which they responded. However, by 8 hours post-LPS, variability within age groups had been reduced and a greater number of significant differences are seen between the young and old cultures. A similar look at the effects of time on the transcriptome of bovine mammary epithelial cells in response to LPS showed low levels of differential expression due to treatment at an early time point (3h) in comparison to a later time (6h), demonstrating that the early response to LPS may be more affected by rapid changes in gene expression making it difficult to generate statistical significance between groups (Gilbert, Cunha et al. 2013).

RNA-Seq analysis also highlighted differences in genes involved in the maintenance of DNA methylation. Older cultures showed higher levels of expression of *DNMT1* the catalytic methyltransferase enzyme responsible for the addition of the

methyl group onto an unmethylated cytosine residue (Chen and Li 2006). Expression levels were also elevated in older cultures for *UHRF1*. This gene product identifies hemi-methylated DNA following mitosis, recruits DNMT1 to the site, and physically flips the methylcytosine residue from the parent strand out of the double helix to sterically allow access of DNMT1 to the demethylated daughter strand (Arita, Ariyoshi et al. 2008, Hashimoto, Horton et al. 2008). The lower levels of *DNMT1* and *UHRF1* expression in the younger cultures potentially suggest that younger cultures may be less engaged in high fidelity maintenance of methylation levels. In contrast, older cultures, having gone through a major developmental shift, are now expressing higher levels of both genes in an effort to maintain DNA methylation. However, differences in DNA methylation due to differential expression of *DNMT1* and *UHRF1* would potentially occur as minor changes rather than active, wholesale modifications in DNA methylation status.

The variable innate immune response in intestinal epithelial cultures had previously been linked to DNA methylation of the NF- κ B inhibitor I κ B α as measured by production of IL-8 following TNF- α exposure (O'Gorman, Colleran et al. 2010). In our experiments the relative increase in LPS-induced IL-8 response following AZA treatment was much greater in younger fibroblasts. This eliminated the differences seen between the young and old cultures in the absence of AZA, suggesting that DNA methylation plays an important role in the development of the immune response. While previous work has focused on determining epigenetic markers that differ between a healthy and diseased population (Andia, de Oliveira et al. 2010, Ishida, Kobayashi et al.

2011, Gillespie, Savic et al. 2012), our study focuses on the regulatory role of methylation across developmental time, potentially providing results that could explain age-dependent differences in pathogen response and perhaps could also have broad implications on animal-to-animal variation in susceptibility to disease. To more directly determine where DNA methylation was changing over time within individuals we performed MIRA-Seq on DNA from fibroblast cultures established from skin biopsies collected from three animals at two ages.

While RNA-Seq data showed major changes in the transcriptome of an individual due to age, MIRA-Seq analysis of the bovine methylome showed fewer alterations than expected. Our methylation analysis identified only twenty 3kb regions that were differentially methylated between young and old individuals. While our comparison within individuals showed little change in methylation levels, this was not due to high variability between individuals. This would appear to indicate that there is a limited change in methylation seen within an individual over the 11 month period investigated within this study. While highly variable methylation levels have been reported in healthy human populations there has been a more limited description of methylation differences within individuals (Bock, Walter et al. 2008, Shen, Qiu et al. 2013). The results from our data suggest that there are a restricted number of wholesale age-dependent changes in the fibroblast methylome that may be detected by the MIRA-Seq analysis. However in light of the results following AZA exposure it appears that more subtle changes, not detectable by MIRA-Seq, are occurring that affect the innate

immune response. The detection of these more subtle changes will require more precise techniques.

A limitation of the current study is the low-resolution nature of MIRA-Seq. Exposure of cultures to AZA demonstrated that DNA methylation is playing a role in the development in the immune response by rescuing the phenotype of young cultures. While MIRA-Seq allows for an overall visualization of the methylome and can discern differences in overall quantity of methylated CpGs down to approximately a 100bp resolution, subtle methylation patterns are missed. This is problematic as DNA regions altered by just a few CpG dinucleotides have been shown to have appreciable effects on gene expression (Stefani, Viana et al. 2013, Sun, Gong et al. 2013). Previous studies that were able to find differences in DNA methylation due to aging and development did so by investigating with a resolution of a single base pair, increasing their power in relation to our data (Martino and Prescott 2011, Raddatz, Hagemann et al. 2013). In addition, work conducted in human epidermal tissues also found that the overall methylome was stable within an individual over time, and alterations were limited to minor, local changes within a 100-150 bp region which again would be lost in our analysis (Raddatz, Hagemann et al. 2013). Future studies would also benefit from an increase in the sample size of each group, which has been shown to be effective in increasing the ability of MIRA-Seq technology to identify minor differences in methylation (Bock, Tomazou et al. 2010), or by using a more sensitive technology such as reduced representation bisulphate sequencing to offer a greater resolution (Bock, Walter et al. 2008).

MIRA-Seq data allowed for the construction of a genome-wide DNA methylation heat map. This map, the first built for the bovine fibroblast methylome, displayed dense regions of methylation interspersed throughout all of the genome. As a composite of the six MIRA-Seq libraries created, we are able to see regions of methylation likely playing major regulatory roles in fibroblast gene expression and differentiation. While there is currently a dearth of information on the methylation status of different bovine tissues, the construction of the fibroblast methylome is an important first step towards a comparative analysis of methylation across cell types within the bovine system.

MIRA-Seq analysis of our samples was able to confirm several important phenomena seen previously in other target species in terms of DNA methylation's relationship to gene expression, and the non-random placement of methylated CpG dinucleotides across the genome. Our data reaffirmed the relationship between gene expression and methylation levels in that heavily methylated genes had a significant tendency towards lower expression levels (Jones 2012). In addition, our data suggested that the placement of methylation along DNA is not a random process and can be altered based upon relation to DNA sequences with distinct functions. More specifically, methylation levels are clearly depleted in the promoter region of genes. Analysis also showed no differences in methylation levels between gene and intergenic regions, suggesting that the promoter region of a gene is highly sensitive to methylation and the one most highly regulated in terms of *de novo* DNA methylation, confirming what has been previously reported (Raddatz, Hagemann et al. 2013).

While RNA-Seq technology was able to define major differences in the recognition and response of bovine dermal fibroblasts to LPS, data investigating the role of DNA methylation in this process was inconclusive. The results from this investigation would suggest that major regulatory changes are occurring within the fibroblasts of individuals due to age. As these genes have demonstrated the ability to be regulated due to age alone, it is possible to determine biomarkers from this study to identify malleable genes to focus on that may be manipulated to produce a more ideal phenotype. While our results focused on the innate immune response, it would seem likely that many cellular processes are undergoing similar alterations during development. In addition, our data using AZA as a global de-methylation agent points to DNA methylation as a major player in the alteration of cellular responsiveness to LPS. A MIRA-Seq-based genome-wide scan of methylation however was able to find only a handful of candidate regions differentially methylated due to age. These regions require further investigation with a more sensitive tool for measuring methylation levels on a larger sample size.

4.5 Conclusions

The LPS-induced transcriptome of bovine dermal fibroblasts is greatly altered due to age, with epigenetic regulation playing a potentially important role. Using multiple cultures from single individual would appear to give researchers an ideal model for investigating not only the role of aging in disease but also general mechanisms controlling the innate immune response. By determining effectors altering

TLR signaling within an individual, this data may be applicable to finding variations within a population in relation to variation in phenotypic response to pathogens.

4.6 Materials and Methods

4.6.1 Animals and Experimental Design

All experiments were performed in accordance with approval of the Institutional Animal Care and Use Committee at the University of Vermont. Three pairs of dermal fibroblast cultures, collected from the same animals at 5 and 16 month of age, were selected from a previously described collection of cultures obtained from 15 Holstein heifers [27]. Fibroblast isolation from the 15 animals and their ability to produce IL-8 in response to LPS has previously been described (Green, Kandasamy et al. 2011). The selected culture pairs for the current experiment came from three mid-level responding animals.

4.6.2 Fibroblast Epigenetic Treatment and LPS Challenge

Following recovery from cryopreservation and expansion in a T-75cm² flask, cells were trypsinized and seeded in 6-well culture plates at 1.25×10^5 cells/mL in a total volume of 2 mL. Growth media consisted of DMEM with 5% fetal bovine serum (Thermo-Scientific, Waltham, MA), 1% insulin-transferrin-selenium (Life Technologies, Carlsbad, CA), and 1X penicillin/streptomycin (Thermo-Scientific) at 37°C with 5% CO₂. Cells undergoing epigenetic treatment were exposed to 5-aza-2'deoxyctidine (AZA; Sigma, St. Louis, MO) to achieve DNA demethylation. Cells undergoing DNA demethylation were cultured for 96 hours in the presence of 10 µM

AZA. Control cells were grown for 4 days with comparable amounts of the AZA vehicle (PBS) added. Dosages and exposure time to AZA were modeled after previous experiments conducted that displayed low cytotoxicity from treatment in conjunction with effective epigenetic remodeling at similar or lower concentrations than those used in our trials (Takahashi, Sugi et al. 2009, Tsai, Li et al. 2012, Duijkers, Menezes et al. 2013)

Following epigenetic modification, cells were washed 3X with PBS and exposed to LPS for 24 hours. LPS treatment consisted of growth media supplemented with 100 ng/mL of ultra-pure LPS isolated from *Escherichia coli* O111:B4 (Sigma-Aldrich). Following appropriate exposure time, media was removed, spun at 500 x g for 5 minutes to remove cell debris, and immediately stored at -20°C until further analysis.

4.6.3 Quantification of IL-8

The concentration of IL-8 in fibroblast conditioned media was quantified by a custom sandwich ELISA previously described (Kandasamy, Green et al. 2011) using mouse anti-bovine IL-8 monoclonal antibody (clone 170.13 generously gifted by S. Maheswaren, University of Minnesota) and biotinylated goat anti-human IL-8 antibody (R&D Systems Inc., Minneapolis, MN) as capture and detection antibodies, respectively, and recombinant bovine IL-8 (Thermo Scientific, Rockford, IL) as assay standard. The detection limit of the assay was approximately 300 pg/ml.

4.6.4 RNA-Seq

Additional aliquots of the fibroblast cultures that were collected at 5 and 16 months of age from the same three animals were revived from cryopreservation,

expanded, and seeded in duplicate wells of 6-well culture plates at 1.25×10^5 cells/mL in a total volume of 2 mL. After two days the cultures were exposed to LPS (100 ng/ml) 0, 2, and 8 hours in the presence of LPS. Total mRNA was collected using the 5-Prime PurePerfect RNA Purification Kit (Gaithersburg, MD). Resulting RNA was quantified and assessed using a Qubit Spectrofluorometer (Life Technologies Carlsbad, CA) and an Agilent Bioanalyzer 2100 (Agilent technologies Santa Clara, Ca) to ensure that all samples had an RNA integrity number (RIN) value of 8 or greater. Library construction was carried out using Illumina TruSeq RNA Sample Prep LT version 2 (Illumina Inc. Boston, MA). Briefly, 500 ng of total RNA was PolyA enriched using magnetic beads followed by reverse transcription using Superscript II (Invitrogen). cDNA was then fragmented, end repaired, and adenylated followed by a ligation of Illumina adaptors with a unique adaptor sequence (barcode) for each sample. PCR amplification was performed using Illumina Reagents followed by quantification and assessment of quality (as described above) along with high accuracy qPCR quantitation (KAPA Biosciences kit # 4824; Barre, VT). All DNA clean-up steps were performed with the AMPure XP Magnetic Beads (Beckman Coulter). Sequencing was performed using 12pM/flow cell lane on an Illumina CBOT for flow cell cluster generation and the HiSeq1000 for sequencing by Synthesis equipped with the HiSeq Control and sequence Analysis Software. The 18 samples were multiplexed across 7 lanes of a flow cell and sequenced as a single end 100bp read.

Raw sequence reads were filtered to eliminate reads that had a median quality (Q) score below 20, more than 3 uncalled bases, or were less than 25 bp following

trimming and aligned to the reference UMD v 3.1 bovine genome using the software package NextGENe v. 2.3.4 (Softgenetics, State College, PA). Alignment parameters required >85% of the each read's length to map to the reference sequence for it to be considered a mapped read. After reads were mapped with NextGENe, total raw read counts for each gene were generated. These read counts were used for further statistical analysis to determine differentially expressed genes as described below.

4.6.5 DNA Isolation and Methylated CpG Island Recovery Assay (MIRA-Seq)

MIRA-Seq libraries were created to investigate genome wide methylation levels in fibroblasts from the 3 individuals at 5 and 16 months of age (n=3/age). 1.5 µg of genomic DNA was isolated from fibroblasts having no previous LPS exposure using the 5-Prime (Gaithersburg, MD) Pure Perfect Archive DNA Extraction kit and sonicated in 150 µl of water for 10 minutes with alternating cycles of 30s on followed by 30s off using a Diagenode Bioruptor 300 (Denville, NJ). Sonicated DNA was purified using the Qiagen MinElute PCR Purification kit. Fragmented DNA then underwent end repair using New England Biolabs (Ipswich, MA) NEBNext End Repair Module following manufacturer's instructions. End-repaired DNA was recovered with AMPure XP Beads (Beckman Coulter, Pasadena, CA) per manufacturer's instructions and then dA-tailed using the New England Biolab NEBNext dA-Tailing Module. Following subsequent cleanup using the AMPure XP Beads, universal adapters were ligated onto the DNA fragments using T4 DNA ligase (NEB). Barcoded adaptors were then attached with the NEBNext Multiplex Oligos for Illumina kit (NEB) followed by purification using AMPure XP Beads.

Next, MIRA pulldown was performed using the Methyl Collector Ultra Kit (Active Motif, Carlsbad CA) per manufacturer's instructions. The methylated DNA rich library DNA was then run out on a 1% agarose gel with 1% SYBR Green and fragments of approximately 300-700bp in size were excised. DNA was then eluted from the gel using the Qiagen MinElute Gel Extraction kit.

PCR amplification of the libraries was conducted using the NEBNext DNA Library kit universal adaptors with Amplitaq Gold with GeneAmp (Life Technologies, Carlsbad, CA). Cycling conditions were: initial denaturation at 98° C for 30 seconds; then 14 cycles consisting of denaturation at 98° C for 10 seconds, annealing at 65° C for 30 seconds and extension at 72° C for 30 seconds with a final extension of 72° C for 5 minutes. PCR products were cleaned using the AMPure XP Beads and run on a 1% agarose SYBR Green gel to remove excess primers as previously described. The DNA library was excised from the gel then purified using the Qiagen MinElute Gel Extraction kit. Each library was tested for enrichment of methylated DNA by performing PCR using a primer pair designed to amplify a known methylated gene (LIT1) as a positive control as well as a primer pair that amplifies an unmethylated gene (MP68) to ensure adequate depletion of unmethylated DNA. LIT1 primer sequences were 5'-TGCTCTGGACGTGGTCCGCCTGG-3' for forward sequence and 5'-CCGGGCGACCGTGGCGACCT-3' for reverse. MP68 sequences were 5'-GGGCCTGGGCCTTGCCCTCA-3' for forward and 5'-TGCAAAAGGCTAGCTGGCTGCAAT-3' for reverse. Sequencing of the libraries was performed using a 12pM/lane bridge amplification on an Illumina CBOT for flow

cell cluster generation and the HiSeq1000 for sequencing by Synthesis equipped with the HiSeq Control and sequence Analysis Software.

Raw sequence reads were filtered to eliminate reads that had a median quality (Q) score below 20, more than 3 uncalled bases, or were less than 25 bp following trimming and aligned to the reference UMD v 3.1 bovine genome using the software package NextGENe v. 2.3.4 (Softgenetics, State College, PA). Alignment parameters required >85% of the each read's length to map to the reference sequence for it to be considered a mapped read. After reads were mapped with NextGENe, total raw read counts for each gene were generated. These read counts were used for further statistical analysis to determine differentially expressed genes as described below.

4.6.6 Analysis of RNA-Seq and MIRA-Seq

RNA-Seq and MIRA-Seq data were analyzed by the well-established statistical methods employed by edgeR in the R software package (version 3.0.1). As an initial step, genes with low read counts, defined as at least one mapped read per million mapped reads (counts per million; CPM) in less than 50% of the samples being compared, were eliminated. For example, comparison of young and old cultures at a given time point employed analysis of n=3 samples/group, so at least 3 samples needed a CPM equal to or greater than 1 to be considered for analysis.

Comparison of young and old animals at different time points for RNA-Seq was conducted using a paired generalized linear model likelihood-ratio test. Paired analysis was also used to determine the response to LPS (0h vs. 2h, and 0h vs. 8 h post-LPS) as well to identify differentially methylated regions (DMRs) in the young and old cultures.

DMR identification was performed using several different comparisons. First, the genome was broken into 3kb regions to allow for an overall scan of the bovine fibroblast methylome. Secondly, gene promoter regions (-2500 to +500bp from the transcription start site) were analyzed to determine specific differences seen in these regions. Raw p-values were adjusted to account for multiple comparisons using the Benjamini-Hochberg method (Benjamini and Hochberg 1995).

When analyzing the effects of age on the LPS response, genes were considered differentially expressed if they passed the false discovery rate (FDR) < 0.05 and fold-change (FC) ≥ 2 thresholds. To determine the effects of LPS on gene expression, cultures at the 2 and 8-hour time points were compared to 0h cultures. When analyzing differential DNA methylation levels, MIRA-Seq data was considered different between group if they passed the FDR < 0.1 and FC ≥ 2 thresholds. The Database for Annotation, Visualization and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov>) was used for functional annotation and analysis by uploading the official gene symbol of statistically significant genes (FC ≥ 2 ; CPM > 1 ; FDR < 0.05 for RNA-Seq, and FC ≥ 2 ; CPM > 1 ; FDR < 0.1 for MIRA-Seq). Finally, genome wide methylation was visualized as a composite of the six MIRA-Seq libraries created using the free software package, Circos (Krzywinski, Schein et al. 2009).

4.6.7 Quantitative Real-Time PCR

A subset of genes identified by RNA-Seq analysis was selected for expression analysis by quantitative real-time PCR (RT-PCR) using oligonucleotide primers specific to *toll-like receptor (TLR)-4*, *IL-8*, *tumor necrosis factor (TNF)- α* , and *cluster*

of differentiation (CD)-14 (Table 1). The same RNA samples from the genome-wide expression study were used. First-strand cDNA synthesis was conducted using the Improm-II Reverse Transcriptase Kit (Promega). Messenger RNA expression was quantified by RT-PCR with a CFX96 Real-Time Instrument (Bio-Rad, Hercules, CA) using PerfeCTa SYBR Green Super-Mix, Low ROX kit (Quanta Biosciences). Cycling conditions were: initial denaturation at 95° C for 2 minutes; then 40 cycles consisting of denaturation at 95° C for 15 seconds, annealing at 60° C for 30 seconds and extension at 72° C for 1 minute. Melt curve analysis was also performed to check amplification of the desired gene product. The *β-actin* gene was used as reference gene for normalization procedure. Cycles to threshold (Ct) were calculated for each sample and analyzed using the ΔC_t method with fold change being $2^{\Delta\Delta C_t}$.

4.6.8 Analysis of RNA-Seq and MIRA-Seq Relationship

To determine the relationship between DNA methylation and gene transcription levels, the average reads per kilobase per million matched reads (RPKM) from the RNA-Seq and MIRA-Seq of the six young and old cultures was investigated. Values were calculated for promoter methylation levels as well as for annotated genes (as defined by the UMD v3.1 genome) from both the MIRA-Seq data and the RNA-Seq data at 0, 2, and 8 hours post-LPS exposure. The relationship of mRNA transcription levels and DNA methylation was then determined by a two-tailed Fisher's exact test in the R software package in which high or low methylation levels were investigated for an association to either high or low RNA expression levels. For gene expression RPKM values, length was calculated as the cumulative size of the gene exons, while for gene

methylation, gene body length was the total size of both intronic and exonic segments. All values were normalized to library and transcript size by conversion of read counts into RPKM values. RNA-Seq RPKM values were binned into either high or low levels at a cutoff of RPKM=5 while MIRA-Seq RPKM values were divided into high or low levels at RPKM=0.5.

To determine whether the type of genomic region assessed had an affect on DNA methylation levels, average RPKM was calculated for gene promoters, gene bodies, and intergenic regions. Gene body and intergenic regions were determined by annotation from the UMD version 3.1 bovine genome, while gene promoters were defined as -2500 to +500 bp of a gene transcription start site. To determine differential methylation levels based upon genomic location, a one-way ANOVA with a Bonferroni post-test for multiple comparisons was run.

4.6.9 Statistical Analysis

IL-8 protein production was analyzed between groups (young vs. old) using a paired Student's t-test (Graph Pad Prism 6.0). Analysis of real time gene expression data was conducted using a two-way ANOVA model with repeated measures (Graph Pad Prism 6.0). Comparisons with $P < 0.05$ were considered statistically significant within experiments.

Additional Files

Additional files are available upon request from Dr. David Kerr at david.kerr@uvm.edu.

Table 1. Primer pairs used for amplification of target genes by RT-PCR.

Gene	Forward Primer Sequence	Reverse Primer Sequence	Reference
CD14	CTCCAGCACCAAAATGAC	TCCTCTCCCTCTCTTCC	(Sohn, Paape et al. 2007)
IL-8	GCTGGCTGTTGCTCTCTTG	AGGTGTGGAATGTGTTTTATG	(Pareek, Wellnitz et al. 2005)
TNF	TCTTCTCAAGCCTCAAGTAACAAG	CCATGAGGGCATTGGCATAAC	(Bougarn, Cunha et al. 2011)
TLR4	ACTGCAGCTTCAACCGTATC	TAAAGGCTCTGCACACATCA	(Ibeagha-Awemu, Lee et al. 2008)
B-Actin	GCAAATGCTTCTAGGCGGACT	CAATCTCATCTCGTTTTCTGCG	(Pareek, Wellnitz et al. 2005)

Table 2. Response to LPS as measured by increase in expression of immune associated genes as compared to Hour 0 post-LPS.

Gene Symbol	Gene Name	Fold Change*	
		Hour 2	Hour 8
Transcription and Activation Pathways			
BIRC3	Baculoviral IAP repeat-containing protein 3	6.1	4.1
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	36.1	10
NFKBIZ	Nuclear factor of kappa-B inhibitor zeta	23.8	4.1
NFKB2	Nuclear factor of κ light polypeptide gene enhancer in B-cells 2	2	2.1
Cytokines and chemokines, growth factors			
CCL2	Chemokine (C-C motif) ligand 2	28.5	26.7
CCL5	RANTES	35.4	48.4
CCL20	Chemokine (C-C motif) ligand 20	2034	1162.3
CSF2	Colony stimulating factor 2	97	6.1
CXCL1	Chemokine (C-X-C motif) ligand 1	2.5	- -
CXCL2	Chemokine (C-X-C motif) ligand 2	43.8	27.1
CXCL5	Chemokine (C-X-C motif) ligand 5	43.8	27.1
IFNB	Interferon beta precursor	2	2
IL1A	Interleukin 1, alpha	86.0	23.5
IL-6	Interleukin 6	83	131.6

IL-8	Interleukin 8	123.7	273.7
SAA3	Serum Amyloid A 3	505.6	2498.3
TNF-a	Tumor Necrosis Factor, alpha	125.2	- -
TNFSF13B	Tumor necrosis factor (ligand) superfamily, member 13 (BAFF)	- -	4.6
Type I IFN-related genes			
IFI44	Similar to Interferon-induced protein 44	- -	15.2
IFIH1	Interferon induced with helicase C domain 1	- -	15.1
ISG15	ubiquitin-like modifier	6.2	63.2
MX2	Myxovirus (influenza virus) resistance 2	- -	121.2
OAS1	2'-5'-oligoadenylate synthetase 1	- -	24.6
OAS2	2'-5'-oligoadenylate synthetase 2	2.8	26.1
TNFSF10	Tumor necrosis factor ligand superfamily member 10 (TRAIL)	- -	15.8

*Data obtained by RNA-Seq and presented as fold induction of the indicated gene at either 2 or 8 hours post-LPS in comparison to expression levels at 0 hours post-LPS. All fold changes shown are FDR<0.05; FC>2; and CPM >1. - - indicates FDR>0.05, FC<2, or CPM<1.

Table 3. Differential expression of immune associated genes at 0, 2, and 8 hours post-LPS exposure of fibroblasts collected at 16 months of age compared to fibroblasts collected at 5 months of age from the same three animals.

Gene	Hour 0	Hour 2	Hour 8
Innate Immune Related Genes			
CCL2	--	7.3	3.2
CCL20	--	18.1	10.1
CCL5	--	10.7	3.4
CD14	5.5	5.7	4.2
IFIH1	--	--	2.1
IL1A	--	2.3	2.8
IL6	--	10.5	7.9
IL8	--	5.8	3.3
MX2	--	--	33.9
OAS1	--	--	12.8
OAS2	--	--	10.6
SAA3	--	--	5.1
TLR2	--	4	2.6
TLR4	2.1	--	2.6
TNF	--	10.4	--
DNA Methylation Associated Genes			
DNMT1	1.6	1.6	1.6
DNMT3A	--	--	--
DNMT3B	--	--	--
UHRF1	3.4	3.3	3.6

Data obtained by RNA-Seq and presented as fold change between young and old cultures. Positive values indicate higher expression in old cultures as compared to low.

Table 4. Differentially methylated 3 Kb regions between young and old individuals as identified by MIRA-Seq and gene expression data for annotated genes.

Fold Difference*	Chr	Genomic Coordinates	Gene**	RNA-Seq Fold Difference		
				Hour 0	Hour 2	Hour 8
4.6	1	72956301-72959300	XXYLT1	1.0	-1.6	-1.0
-5.3	2	25773034-25776033	SP5	1.0	1.0	1.0
3.7	2	70978934-70981933	--	--	--	--
4.7	2	126997334-127000333	ARID1A	1.0	-1.4	1.0
5	2	88469334-88472333	--	--	--	--
4.9	3	116651410-116654409	CXCR7	-1.2	-1.6	-1.3
4.9	5	117094706-117097705	--	--	--	--
4	7	52484546-52487545	--	--	--	--
-4	8	75361987-75364986	ADRA1A	1.0	1.0	1.1
4.2	8	43784287-43787286	DMRT2	-1.1	-1.3	-1.2
6.1	8	101360987-101363986	PALM2	-1.7	-2.4	-1.9
4.7	9	95637251-95640250	--	--	--	--
-4.3	11	8944985-8947984	LOC100337252	5.2	-1.6	2.5
3.7	11	95333785-95336784	--	--	--	--

-3.8	12	80725922-80728921	--	--	--	--
-6.2	17	62367994-62370993	--	--	--	--
-5.3	17	62346994-62349993	--	--	--	--
3.6	19	44563675-44566674	HDAC5	1.1	1.2	-1.5
-4.3	21	65927663-65930662	--	--	--	--
5.9	23	8944593-8947592	ANKS1A	1.3	-1.2	1.1

*Data obtained by MIRA-Seq and presented as fold difference in read count of methylated regions. Positive fold change indicates higher methylation levels in young cultures while negative values are higher methylation in old cultures.

** DMRs with an associated gene indicate that some portion of the 3Kb region falls within an annotated gene, while -- indicates DMRs that are intergenic.

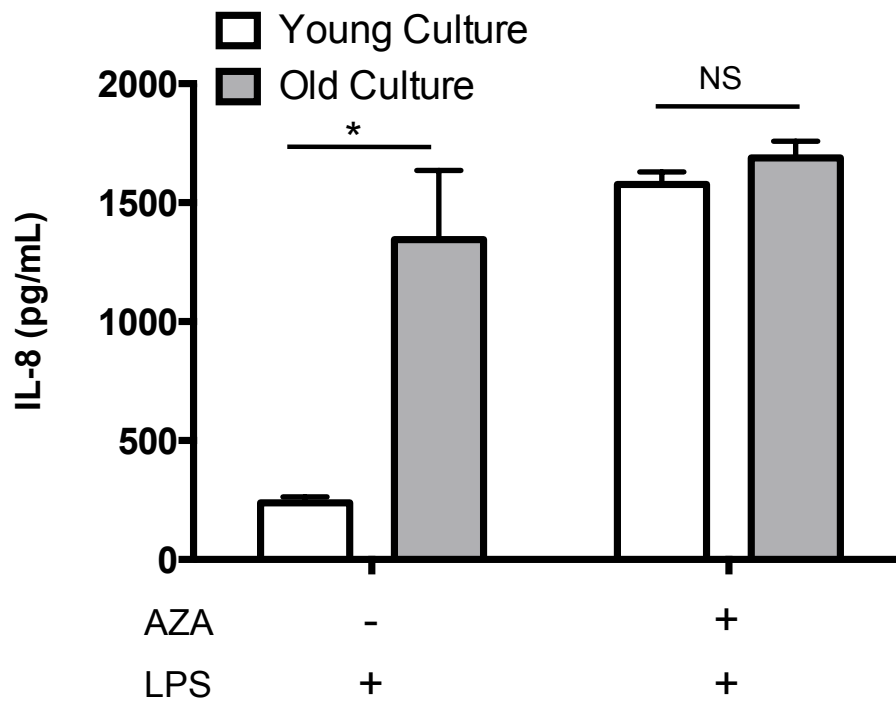


Figure 1. Fibroblast response to LPS under control conditions or following epigenetic modification.

Production of IL-8 by bovine dermal fibroblasts following exposure to LPS (100 ng/ml) for 24 hours after undergoing pre-treatment with media alone or AZA. Cultures collected from the same individual at two different ages (young=5 months of age; old=16 months of age; n=3/group) were investigated. In the absence of AZA, young and old cultures showed differential responsiveness to LPS. However, following exposure to AZA, differences in responsiveness to LPS were lost between the groups. In addition, exposure to AZA significantly increased IL-8 production in response to LPS. IL-8 production in the absence of LPS was not detectable. * indicates $P < 0.05$. All values are mean \pm S.E.M.

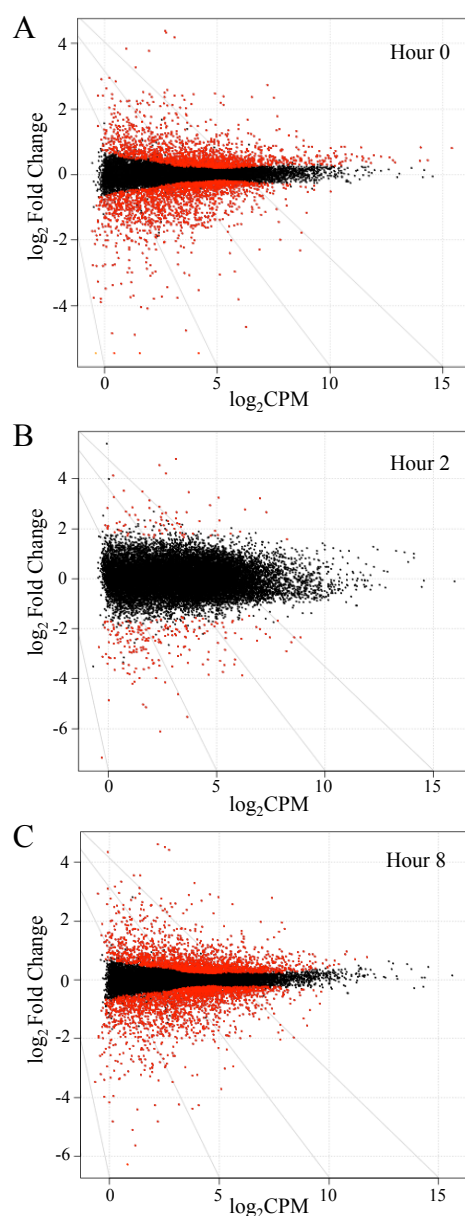


Figure 2. Scatter plots of RNA-Seq analysis.

Scatter plots of indexes analyzed from RNA-Seq for expression level (\log_2 CPM) and differential expression (\log_2 Fold Change) at (A) 0 hours, (B) 2 hours, (C) and 8 hours post-LPS exposure. Positive fold change values indicate higher expression in young cultures while negative values show higher expression in old cultures. Red dots denote $\text{FDR} < 0.05$.

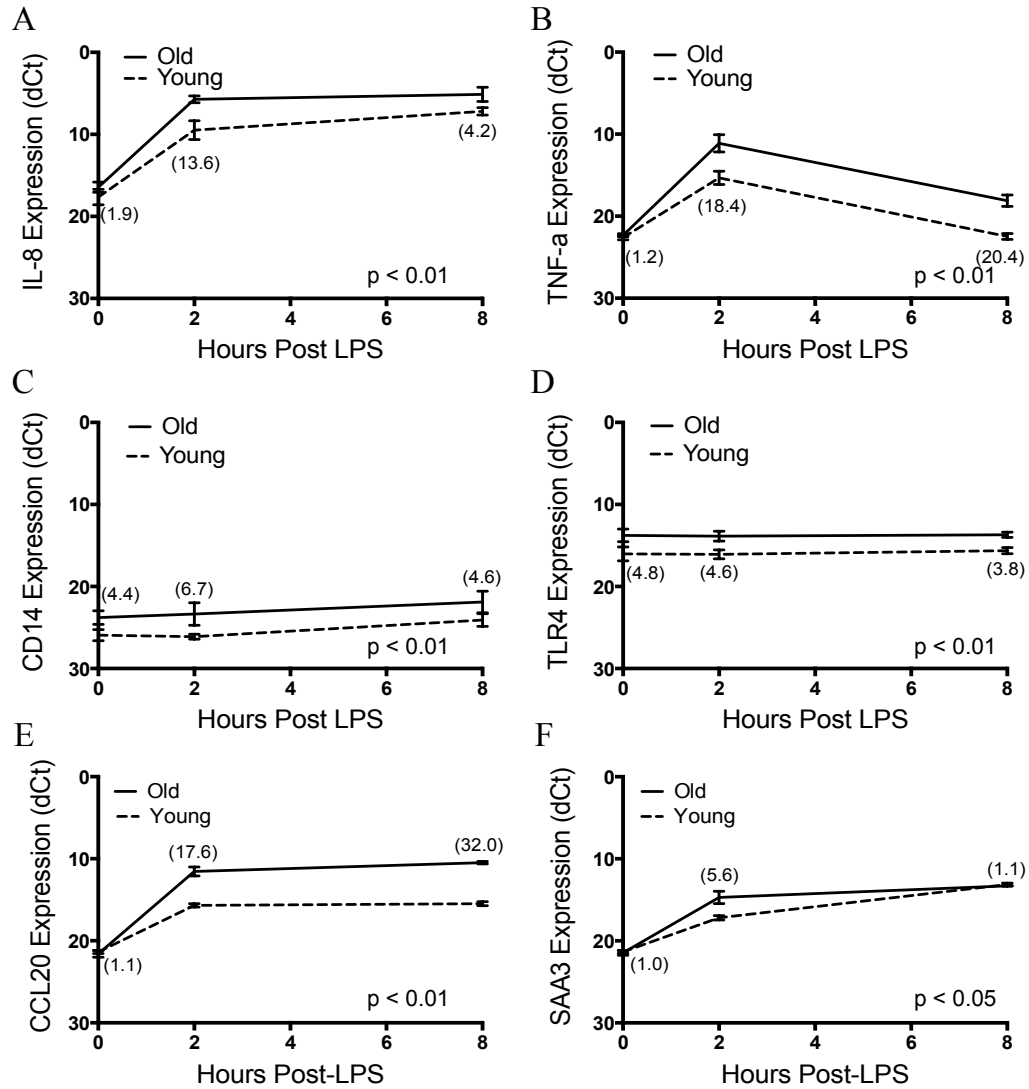


Figure 3. Comparison of young/old and high/low fibroblast response to LPS as measured by RT-PCR.

Comparison of gene expression following exposure to LPS at hours 0, 2, and 8 for young and old cultures. IL-8 (A), TNF- α (B), CD14 (C), and TLR4 (D) mRNA were all targeted. Values are expressed as dCt using β -actin expression as the endogenous control. Fold difference in expression between young and old fibroblasts presented for each time point within parentheses. All values are mean \pm S.E.M. (n=3/group). p-values are presented following analysis using a two-way repeated measures ANOVA.

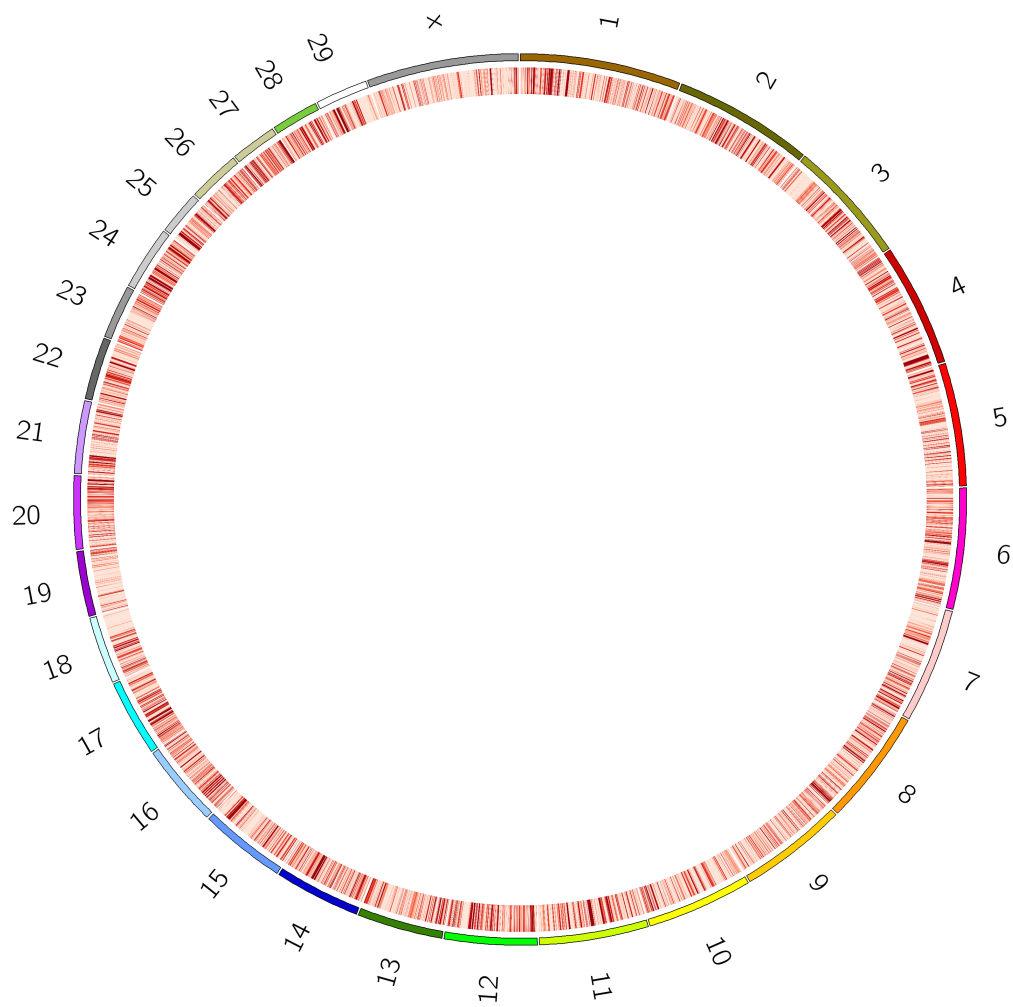


Figure 4. Circos heat map representing methylation levels across the bovine genome. Heat map of the bovine fibroblast methylome. Resolution of construction allowed for a single band to represent a 50Kb chunk of the genome. As indicated, darker red represents heavier levels of methylation within that region.

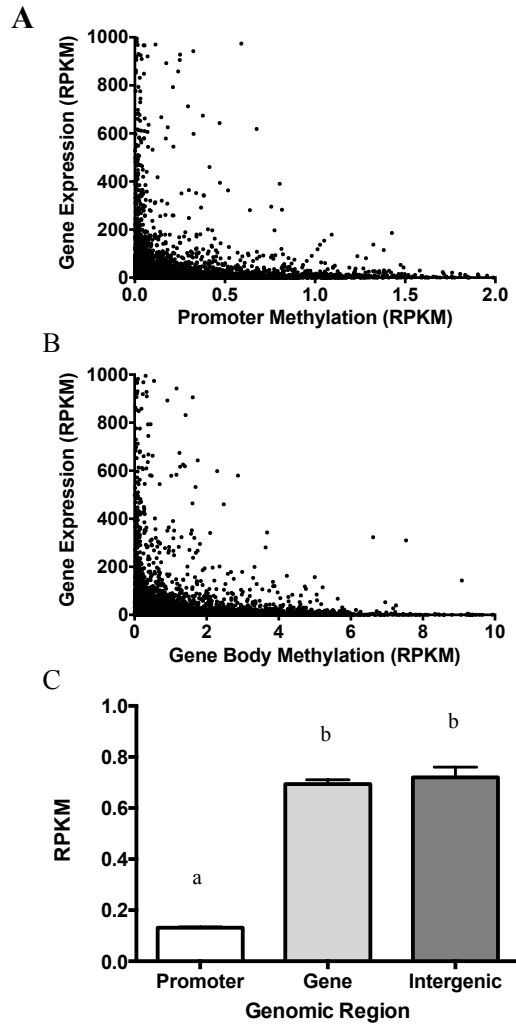


Figure 5. The role of DNA methylation on gene expression and differential methylation levels due to genomic region. Scatter plot showing gene expression at 8 hours post-LPS exposure in relation to the level of DNA methylation at the gene's (A) promoter or (B) within the gene itself. Expression levels for RNA-Seq and methylation data were normalized to gene size and presented as RPKM. Each value represents the average expression of the six young and old cultures investigated. Data obtained by RNA-Seq and MIRA-Seq. (C) Average methylation levels based upon genomic region for young/old MIRA-Seq libraries. Lettering denotes differential levels of methylation as measured by a one-way ANOVA with Bonferonni post-test. All values are mean \pm S.E.M.

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CHAPTER 5:
General Conclusions

Effective control of mastitis has been a difficult goal to attain within the dairy industry. While management practices and aggressive breeding strategies have been able to reduce clinical mastitis rates, there is still much to be done within the field of innate immunity in dairy cows. Currently, a great deal of the difficulty undermining the ability to make thoughtful and effective recommendations to industry workers lies in disagreements within the research community on what represents the most desirable phenotype that should be promoted within a herd. Some researchers (Burvenich, Van Merris, J., Diez-Fraile, & Duchateau, 2003; Rinaldi et al., 2010) have suggested that a strong, rapid response yields the most effective clearance of *E. coli* mastitis, while Kandasamy et al. (2011), have demonstrated that a more mild response is effective in mastitis resolution. In addition, a variety of phenotypic markers, though most notably somatic cell count (SCC) (Haugaard, Heringstad, & Whist, 2013; Heringstad, Klemetsdal, & Ruane, 2000), have been utilized in directed breeding with varying success. This is likely due to the infrequency in which SCC is measured for an individual. Monthly sampling may miss animals that have had an infection with an elevated SCC, but yield a low test value relatively soon after infection due to the acute nature of the infection. An inability to effectively discover a genetic variant linked to disease susceptibility may also be because epigenetic components have previously been ignored, though they may be playing a large role in controlling the expression of genes related to disease resistance.

An array of epigenetically controlled biomarkers that can accurately predict the productivity of an animal early in life would greatly benefit the industry for several

reasons. First, a standardized array that could be implemented within labs to inform on a large-scale level of production would increase the chances of bridging the gap between current scientific understanding and practical implementation. In addition, the use of biomarkers would allow farmers to predict which calves will remain profitable, prior to investment in a heifer that will ultimately lead to profit losses. A recent report following 44 Pennsylvania farms estimated the cost of raising a heifer from birth to freshening at just over \$1,800/animal (Heinrichs et al., 2013). With this being no small investment, the earlier in a heifer's life a farmer may determine likely production, the more likely they will be to run a profitable farm.

The current studies aimed to advance the knowledge of the molecular basis of the TLR4 mediated innate immune response and the potential role DNA methylation may be playing in it. First, how does the innate immune response function early in life, and is this reflective of adult life? By determining the stability of the response to Gram-negative stimuli in calves, we hoped to ascertain how early testing for phenotypic response could be conducted with confidence. Secondly, is there a component other than the primary DNA sequence, namely methylation, which may be playing a role in controlling the magnitude of the response to lipopolysaccharide? By selecting primary cell cultures with variable responses to LPS and comparing DNA methylation from each, could we determine biomarkers acting as prognostic signals of innate immune response?

As has previously been demonstrated in model organisms, we have provided evidence showing that the innate immune response in young calves is dynamic and

increases dramatically during maturation. As the response to LPS increased within an animal, it also did so in parallel with other individuals allowing for a stable ranking of low to high responding cultures within a population. This illustrates the necessity however of age-matched samples in future studies utilizing this model.

Comparative analysis of the innate immune response in dairy animals also displayed the large degree of variability in the response to LPS between individuals. A nearly three-fold difference was seen in the response to LPS as measured by IL-8 production between the lowest and highest responding cultures illustrating the span of individual variation. This difference was confirmed when the lowest and highest responding animals, as determined from *in vitro* responses, were challenged *in vivo* with LPS intravenously and displayed differential production of IL-8 and TNF- α matching predicted difference. This then gave us the ability to work with samples displaying variable phenotypes under two circumstances, low and high responding individuals, as well as low and high responding cultures isolated from the same animal but at different ages.

With the variable immune response described, our work aimed to determine what role, if any, DNA methylation may be playing in creating this altered gene expression. We first utilized global manipulations of DNA methylation by exposing fibroblasts to 5-aza-2'deoxyctidine (AZA) which de-methylates cells by inhibiting DNA methyltransferase proteins. De-methylation using AZA led to a loss of variable response to LPS between young and old cultures collected from the same animal and between age-matched low and high responding animals. To further elucidate

differential gene expression between young and old cultures and its relation to DNA methylation, RNA-Seq was conducted on cultures from the same individuals at different ages and compared to genome wide methylation data produced by MIRA-Seq. Though this comparison was only able to yield 20 differentially methylated regions (DMRs) it gives early insight into how the bovine fibroblast methylome is constructed and its role in gene expression. This included confirming that high levels of methylation directly inhibited gene expression in cows, and that methylation is not random as gene promoters displayed significantly lower levels than the rest of the genome. This is one of the earliest reported methylomes in bovine research.

The research presented here was able to greatly expand the current understanding of DNA methylation in cattle as well as its role in the innate immune system, it also highlights unanswered questions that may act as a roadmap for future work in this field. Our genome wide methylation analysis exclusively utilized MIRA-Seq technology, though this was less effective than we may have hoped. MIRA-Seq uses a protein mediated pulldown technique that leaves the resolution of sequencing at around 100bp, while smaller alterations in methylation may be playing a large role in gene expression. Future studies focusing on methylation and its role in gene expression would benefit from the use of technologies with a greater resolution such as reduced representation bisulfite sequencing or, once more cost effective, whole-genome bisulfite sequencing. While DNA methylation was investigated here extensively, it would also benefit the industry to have a more thorough understanding of the role of histone modifications and the interplay between these epigenetic modifications. Data

presented here would suggest that DNA methylation is a major contributor to the development of the innate immune response, however subsequent studies may benefit from the continual advancement of sequencing technology and the potential cost effective ability to investigate DNA methylation at a single base pair resolution. Identification of consistently modified CpG dinucleotides that could be used as predictive biomarkers would be a major advancement in dairy management practices and in determining which calves left the herd and which remained.

Though the identification of biomarkers would be an important step towards a better understanding of how the dairy innate immune system works, this would still ultimately leave the chances of breeding animals with a desirable phenotype subject to the somewhat random nature of environmental impacts on epigenetics. However, methylation levels have previously shown relative plasticity in response to environmental stimuli suggesting that external cues could be used to drive animals towards a desired response level. Examples of this type of work may be seen in prenatal mice fed varying levels of methyl donors to elicit alternative outcomes in relation to intestinal colitis (Mir et al., 2013), or the reduced productivity of dairy cows that, as calves, had undergone feed restriction (Soberon, Raffrenato, Everett, & Van Amburgh, 2012). The goal of work in this field should then focus on establishing environmental conditions that would increase the likelihood of producing calves with a desired phenotype. If this were to be accomplished, the environmental requirements allowed for continued animal welfare along with a favorable cost to benefit ratio then could drive the dairy industry towards an increased profit margin. Though there is still much

work to be done within the dairy industry to better understand the regulation of the innate immune response and how to identify an individual with heightened disease resistance, a great deal has been accomplished.

APPENDIX A:

MIRA-Seq Data from Low and High Responding Animals.

Experimental Design and Rationale

A large degree of variation exists in the innate immune response of bovine dermal fibroblasts (B. B. Green, Kandasamy, Elsasser, & Kerr, 2011; Kandasamy, Green, Benjamin, & Kerr, 2011), suggesting potential genetic or epigenetic components influencing phenotype. Previous work on these low and high responding cultures has suggested that DNA methylation may be a key player in the variability described, as global demethylation using 5-aza-2'deoxyctidine was able to eliminate differences between individual cultures (Benjamin B. Green & Kerr, 2014). To determine whether DNA methylation levels varied between low and high responding cultures, methylated island CpG recovery assay sequencing (MIRA-Seq) was performed on three low and three high responding (n=6) cultures from animals 16 months of age.

DNA Isolation and Methylated CpG Island Recovery Assay (MIRA-seq)

For MIRA-Seq library construction, genomic DNA was isolated using the 5-Prime (Gaithersburg, MD) Pure Perfect Archive DNA Extraction kit. 1.5 ug of genomic DNA was sonicated in 150 ul of water for 10 minutes total with alternating cycles of 30s on followed by 30s off using a Diagenode Bioruptor 300 (Denville, NJ). Sonicated DNA was cleaned up using the Qiagen MinElute PCR Purification kit. Fragmented DNA then underwent end repair using New England Biolabs (Ipswich, MA) NEBNext End Repair Module following manufacturer's instructions. DNA was cleaned up using the AMPure XP Beads kit (Beckman Coulter, Pasadena, CA) per manufacturer's instructions and then a dA-tail added using the New England Biolab NEBNext dA-Tailing Module. Following subsequent cleanup using the AMPure XP Beads, adapters

were ligated on to the DNA using the New England Biolab T4 DNA ligase. Custom barcoded adaptors (**Table 1**) were ligated on followed by another subsequent cleanup using AMPure XP Beads.

Next, MIRA pulldown was performed using the Methyl Collector Ultra Kit (Active Motif, Carlsbad CA) per manufacturer's instructions, and the methylated DNA rich library was cleaned using the Qiagen MinElute PCR kit. The DNA was then run on a 1% agarose gel with 1% SYBR Green at which point the library was extracted by gel excision to select for fragments approximately 300-700bp in size. DNA was then eluted from the gel using the Qiagen MinElute Gel Extraction kit.

PCR amplification of the libraries was conducted using the NEBNext DNA Library kit universal adaptors with Amplitaq Gold with GeneAmp (Life Technologies, Carlsbad, CA). Cycling conditions were: initial denaturation at 98° C for 30 seconds; then 14 cycles consisting of denaturation at 98° C for 10 seconds, annealing at 65° C for 30 seconds and extension at 72° C for 30 seconds with a final extension of 72° C for 5 minutes. PCR product was cleaned using the AMPure XP Beads and run on a 1% agarose SYBR Green gel to remove excess primers as previously described. The DNA library excised from the gel was then purified using the Qiagen MinElute Gel Extraction kit.

Analysis of MIRA-seq

MIRA-Seq data was analyzed by the well-established statistical methods employed by edgeR in the R software package (version 3.0.1). As an initial step, for each comparison being made, genes with low read counts were eliminated from

analysis. Adequate expression was defined as at least one mapped read per million mapped reads (counts per million; CPM) in the number of samples per group being compared. For example, comparison of cultures at a given time point employed analysis of $n=3$ samples/group, so at least 3 samples needed a CPM equal to or greater than 1 to be considered for analysis.

Comparison of methylation levels was conducted using an unpaired generalized linear model likelihood-ratio test. DMR identification was performed by breaking the genome into 3kb regions to allow for an overall scan of the bovine methylome. Raw P-values were adjusted to account for multiple comparisons using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). When analyzing the effects of response phenotype on the cultures, genes were considered differentially methylated if they passed the $FDR < 0.10$ and fold-change ≥ 2 thresholds. For genomic regions that were differentially methylated and contained an annotated gene, the Database for Annotation, Visualization and Integrated Discovery (DAVID) was used for functional annotation and analysis by uploading the official gene symbol (2-fold expression cut-off; $FDR < 0.10$; $CPM > 1$; <http://david.abcc.ncifcrf.gov>).

To determine whether genomic region had an effect on DNA methylation levels, reads per kilobase per million mapped reads (RPKM) were calculated for samples within gene promoters, gene bodies, and intergenic regions. Gene body and intergenic regions were determined by annotation of the UMD version 3.1 bovine genome, while gene promoters were defined as -2500 to +500 bp of a gene transcription start site. To determine differential methylation levels based upon

genomic location, a one-way ANOVA with a Bonferroni post-test for multiple comparisons was run.

MIRA-Seq Results

The 6 MIRA-Seq samples from the low and high responding cultures resulted in an average of approximately 27 million reads per library following quality control. The genome was analyzed by construction of sequential 3kB regions in a similar manner to the comparison described for young and old cultures. Analysis of the 3kB genome scan yielded 843 regions that showed significantly different levels of methylation between groups (FDR < 0.1; CPM > 1; Fold Change \geq 2). Of the 843 DMRs, 259 displayed higher methylation in high responding cultures while 588 regions displayed greater methylation in low responding cultures. 503 DMRs from this comparison were found at least partially within annotated genes (**Supplemental Table 1**). Pathway enrichment analysis using DAVID on the genes implicated as being differentially methylated between low and high responding cultures identified MAPK signaling ($P = 3.0 \times 10^{-2}$) with 11 altered genes (**Table 2**) of which 9 displayed higher methylation in low responding cultures.

MIRA libraries created for the low/high MIRA libraries had RPKM values of 0.30, 0.48, and 1.32 for promoters, gene bodies, and intergenic regions respectively (**Figure 1**). This analysis indicated significant differences in methylation level by region ($P < 0.001$), with the Bonferroni post-test indicating significant differences between each genomic region.

Discussion

These data presents variable methylation seen between bovine dermal fibroblast cultures presenting a low and high response phenotype to LPS. Though this data may be valuable in determining genomic regions in which DNA methylation may be playing a role in variable innate immune response, we currently do not have comprehensive gene expression data available to compare DMRs with. In this, we have several hundred candidate regions that can not be directly linked to physiological variation. Identification of DMRs often includes regions outside of annotated genes, some thousands of base pairs away from a gene with a known function. This makes determining importance of some of these candidate regions quite difficult. A potential method to overcome this, and narrow down the number of regions of interest selected for further investigation is to use DMRs identified from previous MIRA-Seq experiments conducted on young and old fibroblasts which present themselves with a low and high phenotypic response to LPS, respectively (Data are presented in Chapter 4). However, upon further investigation, none of the 20 DMRs identified from the young and old experiment overlapped with the DMRs identified here.

One pathway of interest, following functional annotation of the annotated genes using DAVID is the MAP kinase signaling cascade. The MAP kinase pathway has been implicated in the activation of many processes, of which one is innate immune signaling, though most notably through the JNK and p38 transcription factors, rather than NF- κ B (Newton & Dixit, 2012). Variation in the expression and activation of MAP kinase signaling proteins could potentially lead to differential production of pro-

inflammatory cytokines, such as the ones measured in this study to differentiate low and high responding cultures (Arthur & Ley, 2013). More work should be conducted on these cultures, along with others described as having displayed a differential response phenotype to LPS in relation to MAP kinase signaling and its potential role in this phenomenon.

In conclusion, low and high responding individuals display a high level of variability in DNA methylation levels as described by MIRA-Seq. This variation could prove to be insightful into determining mechanistic controls of innate immune signaling, however the lack of expression data limits its value as currently presented. These data however could be paired with other DNA methylation experiments that does include gene expression data and identified DMRs could be compared with others identified. Any overlap in the regions identified would act as a strong indicator of potential function in the TLR4 signaling pathway and would warrant further investigation.

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Table 1. Custom barcoded adapters used for MIRA-Seq library construction.

Unique Sequence	Actual Sequence of Adapter
ACGT	/5Phos/CGTAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACG*T
CGTT	/5Phos/ACGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGT*T
GTAT	/5Phos/TACAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTA*T
TACT	/5Phos/GTAAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAC*T
AGCT	/5Phos/GCTAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGC*T
CTGT	/5Phos/CAGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG*T

Table 2. Differentially methylated regions between high and low responding cultures associated with MAP kinase signaling.

Chromosome	Gene Symbol	FDR	Fold Change
19	CACNG1	2.10E-02	2.33
19	CACNA1G	7.40E-02	1.75
22	EGFR	9.80E-02	-2.77
25	MAPK8IP3	3.70E-02	1.87
19	MAP2K4	8.30E-02	1.71
16	MAPKAPK2	4.70E-02	1.79
5	NTF3	6.20E-02	1.80
13	NFATC2	7.20E-03	1.99
3	PRKACB	8.90E-02	2.11
16	PTPN7	8.00E-02	1.83
22	CACNA2D3	5.50E-02	-2.28

Data obtained by MIRA-Seq. Fold change indicates relative read counts between low and high responding cultures. A positive fold change indicates higher methylation in low responding cultures while a negative fold change indicates higher methylation in high responding cultures.

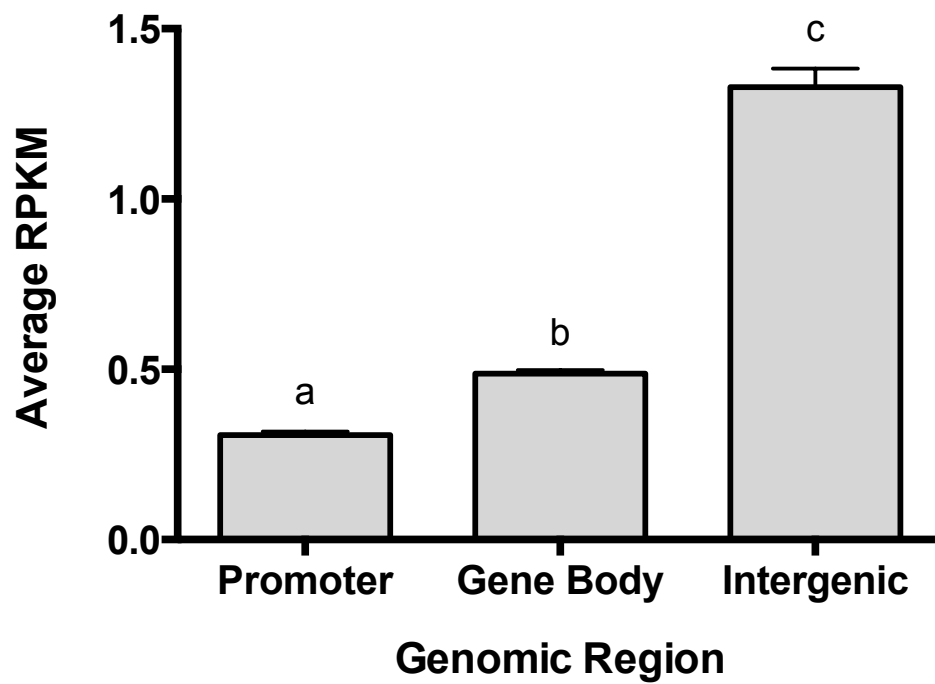


Figure 1. Average methylation levels based upon genomic region for high and low responding cultures as determined by MIRA-Seq.

Lettering denotes differential levels of methylation as measured by a one-way ANOVA with Bonferonni post-test. All values are mean \pm S.E.M.

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